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The role of mannose-binding lectin in health and disease

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Abstract

Mannose-binding lectin (MBL) is a collagenous lectin found in the serum of mammals and birds. In humans, it is believed to play an important role in innate immunity by binding to carbohydrates on the surface of microorganisms and then activating the complement system via MBL-associated serine proteases (MASPs). MBL may also interact directly with phagocytic cells to promote the opsonisation of bacteria and viruses. Serum concentrations of MBL are determined by three structural mutations in the exon 1 region of the MBL2 gene and by polymorphisms in the MBL2 promoter region. Approximately a third of the population have low levels of MBL and appear to have increased susceptibility to infection, especially in children and the immunocompromised. The work presented in this thesis has provided further information on the role of MBL in susceptibility to infection and also provided insights into the binding of MBL to microorganisms and how MBL can influence cell adhesion molecule expression and neutrophil opsonophagocytosis.

Initially, MBL binding to defined lipopolysaccharide (LPS) mutants of *Helicobacter pylori* and LPS and lipid A mutants of *Neisseria meningitidis* was assessed by flow cytometry. Dramatic differences in binding were observed between different mutants indicating the importance of LPS structure in determining MBL binding. Analyses of the LPS structures involved revealed that MBL binding was not merely dictated by the presence or absence of known ligands for MBL in the LPS terminal regions. In addition, four *Mycoplasma* organisms as well as 10 different *Proteus mirabilis* clinical isolates were studied for MBL binding. There was no binding of the lectin to any *Proteus* organisms and A39 strain of *Mycoplasma* whereas *Mycoplasma pneumoniae*, *hominis* and *orale* did bind MBL avidly. All binding was carbohydrate-specific and calcium dependent.

Next two different MBL ELISA procedures were compared and then assessed in parallel with immunonephelometry in order to establish the most accurate assay for measuring MBL concentrations. The AntibodyShop Oligomer MBL ELISA kit was chosen for subsequent studies as the levels determined by this assay correlated well with MBL2 haplotypes. Using this assay, MBL plasma concentrations and MBL2 genotypes were determined in an unselected UK child population

(ALSPAC). Both coding and promoter MBL2 variants were shown to be significantly associated with MBL levels.

In another study three cohorts of patients with meningococcal disease, severe sepsis and cystic fibrosis were investigated. 770 patients with meningococcal disease were genotyped for MBL variants and their haplotype related to susceptibility to the disease and the severity of the disease as assessed by mortality. MBL genotype had only a marginal effect on susceptibility to the infection. However, there was a significant relationship between MBL2 variants and survival from this disease (chi-square test).

In an adult cohort of patients with sepsis, the relationship between MBL2 exon 1 and promoter -221 polymorphisms, plasma levels of the encoded protein, and the incidence and outcome of severe sepsis and septic shock were investigated. Individual plasma levels were variable and increased between days 1 and 7. The mortality rate was higher in those with MBL levels <1000 μ/L (47.2 vs. 22.2%, P = 0.05) (multiple logistic regression analysis). The exon 1 polymorphisms (A/O or O/O) were significantly more common in the patients with severe sepsis and septic shock than in normal healthy adults (54.6% vs. 39.7%, P = 0.001). There was no significant difference in MBL2 genotype or haplotype frequency between survivors and nonsurvivors.

In the third cohort, 260 children were recruited from the paediatric CF clinic at the Royal Brompton Hospital during 2000 and early 2001 for the next study. In this group, low MBL levels were not associated with poor pulmonary function during childhood. There was no significant difference in predicted FEV_1, FVC or the annual rate of decline between high, medium and low-expressing MBL haplotype groups.

The effect of MBL on the expression of adhesion molecules by endothelial cells following bacterial stimulation was also studied. MBL used at physiological concentrations significantly reduced the expression of CD62E by endothelial cells after their incubation with both wild type and *cpsD*$^-$ *Neisseria meningitidis* organisms. MBL did not make any difference to the CD54 expression by HUVECs under similar conditions. The difference in meningococcal LPS structure as well as incubation time did not influence MBL effect on the expression of both adhesion molecules.

The final results chapter describes experiments which show that MBL has an additive effect to other complement pathways during complement deposition on *Staphylococcus aureus*. This led to enhance neutrophil phagocytosis of the bacterium.

The studies presented in this thesis provide further insight into the potential role and mechanisms of MBL in human disease.

Acknowledgment

First and foremost I would like to thank my supervisors Prof Mac Turner and Prof Nigel Klein for their enthusiasm, guidance, advice, support and encouragement during the past years. Their leadership has constantly inspired me to aim for the highest possible standards in research. I would like to thank Mac Turner for his integrity and patience in reading through numerous drafts of my manuscript and Nigel for those hours together, whose input gave me the understanding and skills in order for me to write this thesis.

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Finally, I wish to dedicate this work to my parents, who have always supported me in everything that I have done. In particular my dad, for making me realise that if he

can do what he is doing over the last years without a single complaint and with a smile on his face, then I can try as well.

Declaration

All experimental work presented in this thesis had been done by the candidate unless it is stated otherwise in each individual chapter.

Abbreviations

A	absorbance
AD	Alzheimer's disease
ADP	adenosine diphosphate
AIDS	acquired immunodeficiency syndrome
α_2M	alpha-2-macroglobulin
ASF	airway surface fluid
bp	base pair
C	complement
CD	cluster of differentiation
CD	Crohn's disease
CF	Cystic fibrosis
CF ASF	cystic fibrosis airway surface fluid
CFU	colony forming units
CR1	complement receptor 1
CRD	carbohydrate recognition domain
C1qR	60 kDa collectin receptor
C1qRp	126 kDa C1q receptor
CSF	cerebrospinal fluid
3D	three dimensional
DC	dendritic cells
DIC	disseminated intravascular coagulopathy
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
ECAF	endothelial attachment factor
ECL	enhanced chemiluminescence
EDTA	ethenediamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescent antibody labelled cell sorter
FCS	fetal calf serum
FEV1	forced expiratory volume for 1 second
FFP	fresh frozen plasma

FITC	fluorescin isothiocyanate
FPLC	fast protein liquid chromatography
FVC	forced vital capacity
Fuc	fucose
Gal	Galactose
GalNac	N-galactose-D-acetylamine
GBS	Guillan-Barre syndrome
GC	gonococcal
GDP	guanosine diphosphate
GI	gastro intestinal
Glc	glucose
GlcNac	N-acetyl-D-glucosamine
Gly	glycine
GRE	glucocorticoid responsive element
HBSS	Hank's buffered saline solution
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSE	heat shock element
HUVEC	human umbilical vein endothelial cells
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule 1
Ig	immunoglobulin
IgAN	IgA nephropathy
IMS	industrial methylated spirits
IL	interleukin
I/R	ischaemia-reperfusion
kDa	kilo Daltons
KDO	keto-deoxyoctonate
LOS	lipooligosaccharide
LPS	lipopolysaccharide
M	molar
MAC	membrane attack complex

Man	mannose
MASP	mannose-binding lectin-associated serine protease
MBL	mannose-binding lectin
MD	meningococcal disease
MFI	median fluorescence intensity
NMR	nuclear magnetic resonance
OD	optical density
P	probability
PCR	polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule
RA	rheumatoid arthritis
rhMBL	recombinant MBL
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SIDS	sudden infant death syndrome
SIRS	systemic inflammatory response syndrome
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
SP-A	surfactant protein A
SP-D	surfactant protein D
TBE	tris borate-EDTA
TLR	toll-like receptor
TNF-a	tumour necrosis factor alpha
UC	ulcerative colitis
UHG	universal heteroduplex generator

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Chapter 1 Introduction

1.1. Innate immunity

Immunity to infection is mediated by two general systems: acquired (or adaptive) and innate which has evolved to recognise general characteristics of organisms. Acquired immunity arose early in vertebrate evolution, between the divergence of cyclostomes (lampreys) and cartilaginous fish (sharks) (Fujita *et al.*, 2004). The innate immune system is an evolutionary ancient form, and it is crucial for the first line of defence before the acquired immune system comes into play (Hoffmann *et al.*, 1999). Innate immunity was formerly thought to be a non-specific immune response characterised by phagocytosis. However, innate immunity has considerable specificity and is capable of discriminating between pathogens and self, as proposed in the concept of pattern recognition receptors of host organisms. The skin and other epithelial surfaces such as those lining the respiratory tract, gastro-intestinal tract and genitourinary tract can be considered the most basic defence systems involved in innate immunity. Cells important in the innate immune response include phagocytes (neutrophils, monocytes and macrophages) as well as those which release inflammatory mediators (basophils, mast cells, and eosinophils) and natural killer (NK) cells. There are also a variety of soluble mediators of the innate immune system including the components of the complement cascade(s) - which will be discussed in more detail in section 1.2 - acute phase proteins and cytokines (Janeway and Medzhitov, 2002).
Innate immunity has also been shown to be important in mediating the adaptive immune response (Carroll and Prodeus, 1998; Fraser *et al.*, 1998.)

1.2. Complement

Complement was first described in the 1890s as a heat-labile protein in serum that "complemented" heat-stable antibodies in the killing of bacteria reviewed by Fujita *et al.* (2004). Fifty years later, it was proposed that complement could be activated by bacterial surfaces through an antibody-independent pathway. Now, the complement system, which consists of more than 30 plasma and cell-surface proteins, is known to be a highly sophisticated host-defence system that is engaged both by innate immunity and as one of the main effector mechanisms of

the antibody-mediated response. The main function of complement is to eliminate microorganisms. This is usually done by opsonisation, a process whereby the pathogen is coated with complement components which are then recognised by phagocytes as a signal for engulfment and elimination, and / or lysis of the pathogen by activation of the terminal or lytic pathway forming a structure known as the membrane attack complex. Complement also has other functions, which include the contraction of smooth muscle, release of histamine from mast cells and platelets and increasing vascular permeability.

There are three major pathways of the complement system: the classical pathway, the alternative pathway and the lectin pathway (Figure 1-1). These pathways all converge at the point of generating a C3 convertase, and can all lead to the generation of a membrane attack complex (MAC). The classical and alternative pathways will be briefly reviewed below and the lectin pathway will be discussed in more detail in Section 1.8.1.

1.2.1. The Classical Pathway

The classical pathway was the first to be discovered and requires the presence of IgG or IgM antibodies for initiation, either bound to cell surfaces or as antigen-antibody immune aggregates. These immune complexes can then be recognised by C1q, the recognition portion of the C1 complex similar in structure to mannose-binding lectin, but which binds to the Fc portion of the antibody. Each C1 complex is made up of one molecule of C1q and 2 each of C1r and C1s. Following binding to the immune complex, C1q is able to activate C1r, a molecule with protease activity which can then cleave C1s to create a C1 esterase which is itself able to cleave the next proteins in the cascade, normally C4 and C2.

C4 and C2 both have specific peptide bonds which can be recognised and cleaved by C1 esterase and each generates two fragments – C4a and C4b, and C2a and C2b respectively. The C4b fragment is a reactive molecule that can form covalent bonds with cell surfaces and which also acts as an acceptor for the C2a fragment. This complex, C4b2a is the C3 convertase of the classical pathway, which can activate and cleave C3 into C3a and C3b. The C3b fragment then becomes attached to the cell surface and to C4b2a to form a C4b2a3b. The incorporation of

C3b into this complex alters the specificity of the enzymatic portion (C2a) from C3 to C5 and hence this new complex expresses C5 convertase activity.
Upon cleavage of C5 by its convertase the cascade of the terminal complement components is initiated, sometimes referred to as the terminal or lytic pathway. C5 is cleaved into C5a and C5b and the latter is able to bind C6 and C7 forming the complex C5b67, which can insert into the cell membrane. The formation of this complex leads to the recruitment and attachment of C8, which in turn leads to the recruitment and attachment of multiple C9 molecules which polymerise to form a pore like structure in the cell membrane, $C5b6789_n$, where n can be up to 18 molecules. This structure is also known as the membrane attack complex.

In order to prevent the total consumption of complement components in the serum careful regulation of the classical pathway is necessary. One mechanism of regulation is the activation of C1 inhibitor following C1s activation. This results in C1 inhibitor forming complexes with the C1r and C1s components of C1 molecules, resulting in the dissociation of C1q. C1 esterase inhibitor deficiency (hereditary or acquired angio-oedema) is characterised by the occurrence of subcutaneous swelling in any parts of the skin and the respiratory and gastrointestinal tracts (Gompels *et al.*, 2004). The prevalence of the disease has been estimated at 1/50,000, with no reported bias in different ethnic groups. There is an extreme variability in the symptoms frequency and severity in this disease (Cicardi *et al.*, 1998).
Factor I is also able to regulate the classical pathway, using one of three possible co-factors - C4-binding protein (C4bp), complement receptor 1 (CR1) and membrane cofactor protein (MCP) - to assist in cleaving C4b and thereby resulting in loss of its biological activity. C4bp, CR1 and another membrane-bound protein known as decay accelerating factor (DAF) can also assist in regulation by promoting dissociation of both the C3 and C5 convertases of the classical pathway.

1.2.2. The Alternative pathway

The alternative pathway of complement activation does not require the presence of antibodies for initiation. Instead this pathway depends upon a continual low level

activation of C3 and its possible subsequent activation and deposition onto molecular structures on target cells.

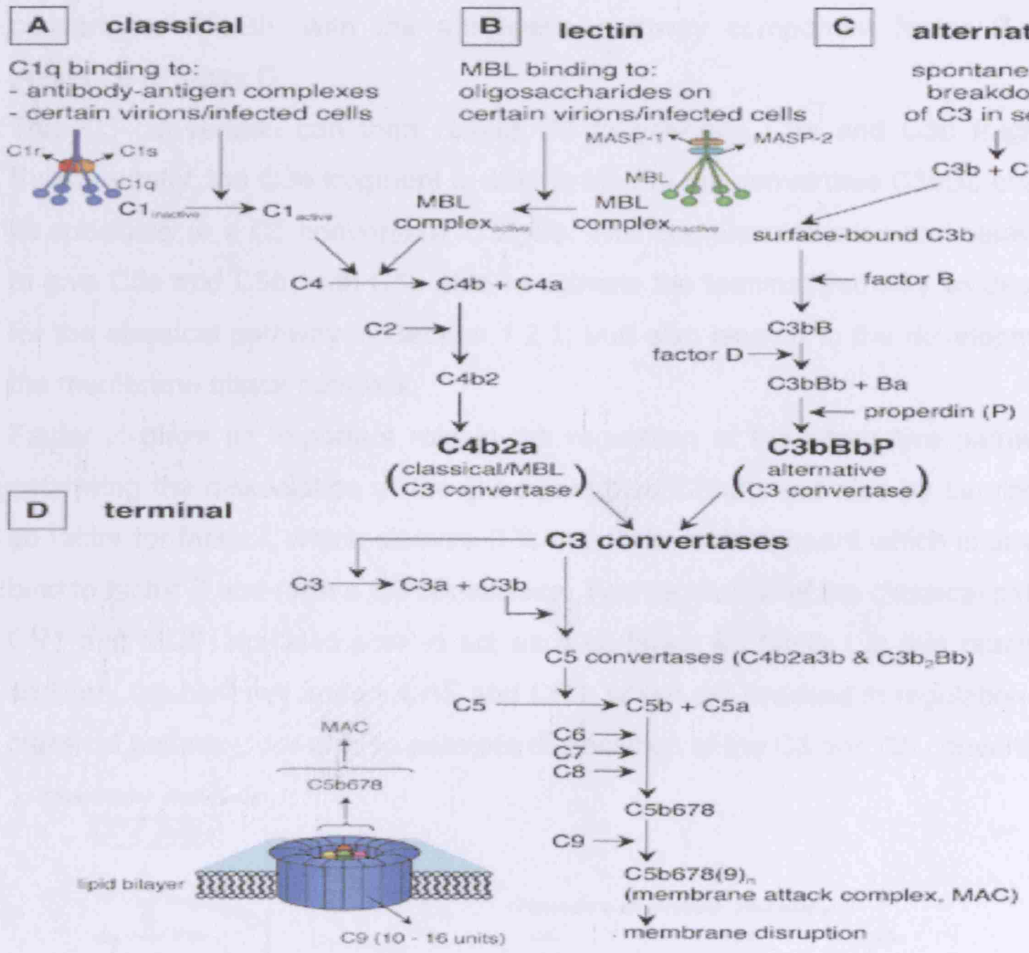


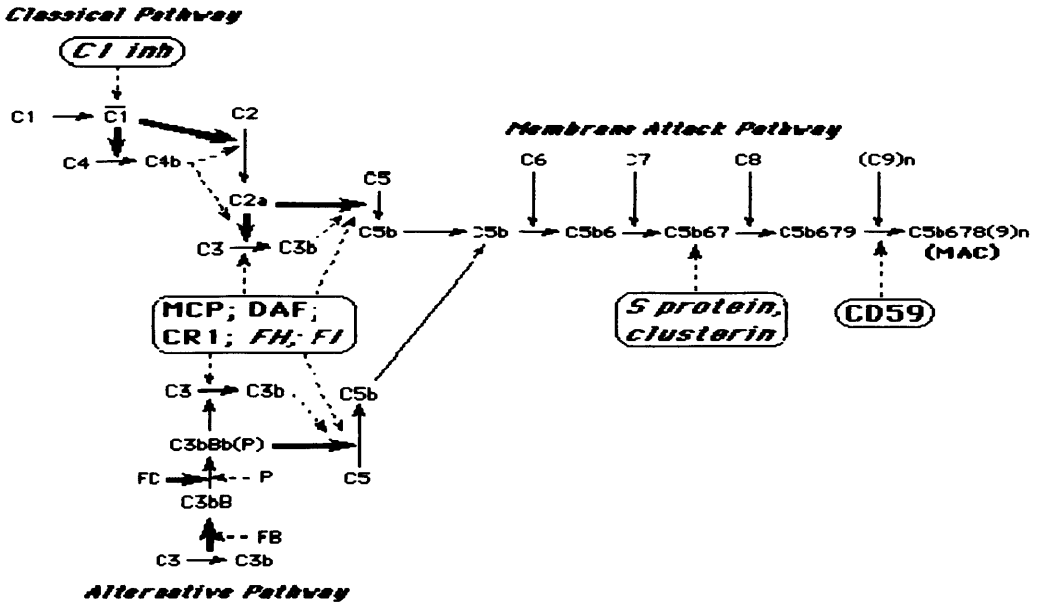
Figure 1-1. Activation of the classical, lectin, and alternative pathways.

The classical pathway is initiated by the binding of the C1 complex to antibodies bound to antigen on the surface of bacteria. The C1 complex consists of C1q and two molecules of C1r and C1s. The lectin pathway is initiated by binding of either of MBL or ficolin, associated with MASP-1, MASP-2, MASP-3, and sMAP to an array of carbohydrate groups on the surface of bacterial cell. As with C1s, MASP-2 is responsible for the C4 and C2 activation, leading to generation of the same C3 convertase as the classical pathway. The alternative pathway is initiated by the low-grade activation of C3 by hydrolysed C3[C3(H_2O)] and activated factor B (Bb). The activated C3b binds factor B (B) which is cleaved into Bb by factor D (D) to form the alternative pathway C3 convertase, C3bBb.
Reproduced from Favoreel *et al.* (2002).

C3 can be naturally cleaved to C3b by serum proteases or activated to C3i (C3 with a hydrolysed thiolester bond but without the loss of the C3a fragment) by small nucleophiles or water. C3i, also known as C3 (H_2O) is able to form a C3 convertase, C3bBb, with the alternative pathway component factor B in the presence of factor D.

This C3 convertase can then cleave C3 to generate C3a and C3b fragments. Subsequently, the C3b fragment is able to bind to the convertase C3bBb changing its specificity to a C5 convertase, $C3b_2Bb$. This complex activates and cleaves C5 to give C5a and C5b, with C5b able to activate the terminal pathway as described for the classical pathway in Section 1.2.1, and also leading to the development of the membrane attack complex.

Factor H plays an important role in the regulation of the alternative pathway by catalysing the dissociation of the C3 convertase C3bBb and also by serving as a co-factor for factor I, which cleaves C3b into iC3b, a component which is unable to bind to factor B and form a C3 convertase. Two regulators of the classical pathway, CR1 and MCP, are also able to act as a co-factor for factor I in this reaction. In addition, the host cell factors DAF and CR1, which are involved in regulation of the classical pathway, are able to promote dissociation of the C3 and C5 convertases.



The complement pathway & its regulation. Enzymatic steps are represented by heavy arrows. Regulatory proteins are boxed; membrane regulators in plain text, fluid phase regulators in italics. Abbreviations: C1 inh, C1 esterase inhibitor; MCP, membrane cofactor protein; DAF, decay accelerating factor; CR1, complement receptor 1; FH, factor H; FB, factor B; FD, factor D; P, properdin; MAC, membrane attack complex.

1.3. Pattern recognition receptors

The expression of innate immunity relies on a well-defined, limited, unchanging pool of germline-encoded pattern-recognition receptors (PRRs) that can bind to highly conserved structures known as pathogen-associated molecular patterns (PAMP), which are commonly present across large groups of invasive microorganisms. These receptors are expressed with identical specificities on many effector cells of the innate immune system (e.g., macrophages and dendritic cells) or are secreted in the serum (Beutler, 2004).

PRRs can be divided into three classes: signalling, endocytic, and secreted (Medzhitov and Janeway, 2002). Mannose-binding lectin (MBL), ficolins, C-reactive protein and serum amyloid protein (SAP) are all secreted pattern-recognition molecules which can function as opsonins to enhance phagocytosis and activate complement (Janeway and Medzhitov, 2002).

An important family of pattern-recognition receptors are the toll-like receptors (TLRs) found on macrophages, dendritic cells and epithelial cells. Toll-like receptors are type I transmembrane proteins involved in innate immunity by recognising microbial conserved structures (Akira, S. and H. Hemmi, 2003). It has been estimated that most mammalian species have between ten and fifteen types of Toll-like receptors (Du *et al.*, 2000; Chuang *et al.*, 2000; Tabeta *et al.*, 2004). The function of the TLRs was discovered by Beutler and colleques (Poltorak *et al.*, 1998).

Recent studies have shown that TLR3 recognizes dsRNA, a viral product, whereas TLR9 recognizes unmethylated CpG motifs frequently found in the genome of bacteria and viruses, but not vertebrates. TLR4 was identified as an LPS recognition molecule (Poltorak *et al.*, 1998).

Pattern-recognition receptors which are found on the cell surface include CD14, a co-receptor for the Toll-like receptors (TLRs), the macrophage mannose receptor and the macrophage scavenger receptors both of which promote the attachment of microorganisms to the phagocyte resulting in internalisation and destruction (Fraser *et al.*, 1998). The carbohydrate recognition domain of the mannose receptor is able to recognise and bind to mannose and fucose and, to a lesser degree glucose and *N*-acetylglucosamine. This recognition profile is quite different

to that of the scavenger receptor which binds to cell wall components such as LPS, peptidoglycan and lipoteichoic acid.

Collectins are an example of humoral molecules which recognize pathogen-associated molecular patterns (Holmskov *et al.*, 2002). In order to eliminate micro-organisms, the collectins cooperate with phagocytes and humoral factors, including complement (Holmskov *et al.*, 2002).

1.4. The collectin family

The C-type lectins are pattern recognition molecules of the immune system, which are neither immunoglobulins nor enzymes and contain a Ca^{2+} dependent carbohydrate recognition domain. The C-type lectins can be divided into six groups according to their properties: the proteoglycan core proteins, the hepatic type II receptors, the macrophage mannose receptor, the natural killer cell receptors, the selectins and the collectins. The collectins are C-type lectins characterised by both a collagenous domain and a lectin domain and have been shown to be of importance in the innate immune response (Sastry *et al.*, 1993; Turner *et al.*, 1996; Lu *et al.*, 1997).

There are seven members of the collectin family, three of which are serum proteins: mannose-binding lectin, bovine conglutinin and bovine collectin-43 (CL-43); and two of which are lung surfactant proteins (SP-A and SP-D) (reviewed by Holmskov *et al.*, 1994). The sixth and seventh members of the family, collectin liver 1 (CL-1) and collectin placenta 1 (CL-P1) have only been recently described and differ from all of the others as they are not secreted (Ohtani *et al.*, 1999; Ohtani *et al.*, 2001). All of the collectins consist of polypeptide chains, which trimerise to form subunits, with each having an N-terminal cysteine rich region, a collagen-like region, a neck region and a C-terminal carbohydrate recognition domain (CRD). These subunits can then form higher order oligomers depending upon the particular collectin (Figure 1-2). The collectins bind to different bacteria, viruses, and fungi (Holmskov *et al.*, 2002). MBL upon its binding activates complement, while SP-A and SP-D does not (Holmskov *et al.*, 2002). MBL, SP-D, SP-A and CL-P1 mediate phagocytosis (Gaynor *et al.*, 1995). SP-A and SP-D bind to apoptotic neutrophils and enhance their clearance by alveolar macrophages (Schagat *et al.*, 2001).

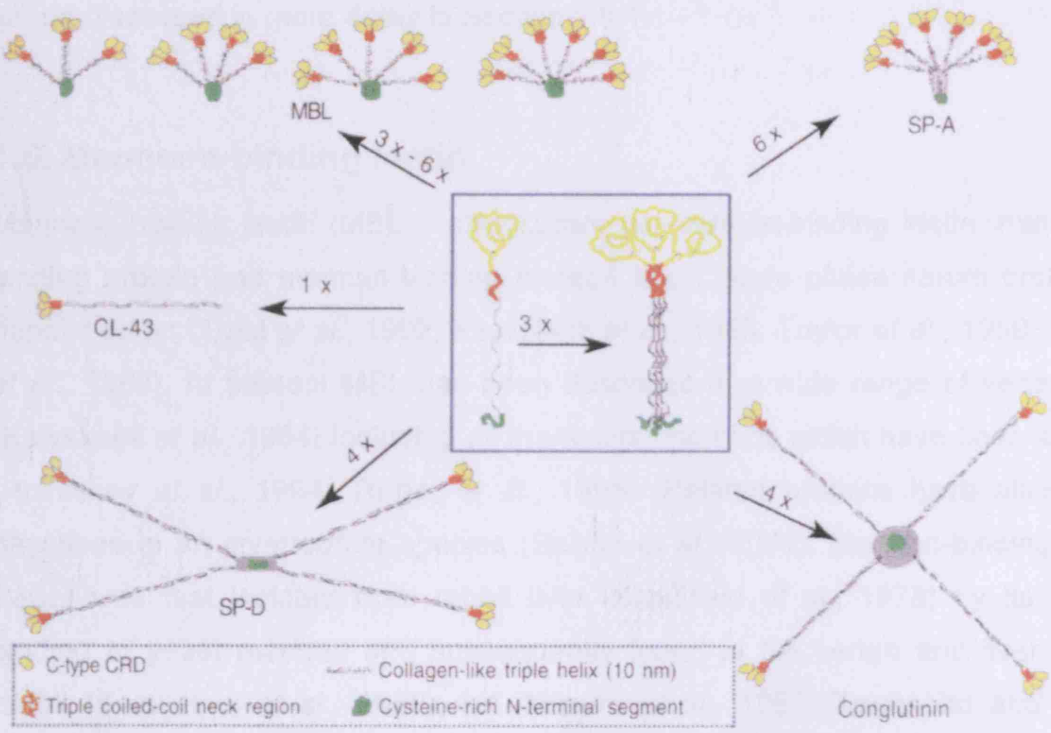


Figure 1-2. The collectin family of proteins.

Diagrammatic representations of five members of the collectin family are shown, illustrating their structural similarities to each other. The sixth and seventh members of the family are collectin liver 1 (CL-L1) and collectin placenta 1 (CL-P1). Taken from Lu *et al.* (1997).

Ficolins are serum pattern-recognition molecules that have some homology to collectins but have a collagen-like domain and a fibrinogen-like domain. In human serum two ficolins have been identified, L-ficolin and H-ficolin (Hakata antigen). They are associated with MBL-associated serine proteases (MASPs) and activate complement (Matsushita *et al.*, 2000; Matsushita *et al.*, 2001). Recently, it had been reported that L-ficolin binds to lipoteichoic acid, a cell component found in all Gram-positive bacteria (Lynch *et al.*, 2004). Another ficolin, termed M-ficolin or P-35 related protein, whose mRNA is found in leukocytes and lung, is not considered to be a serum protein (Lu *et al.* 1996; Endo *et al.* 1996). M-ficolin, which can associate with MASPs, activates complement via lectin pathway and specifically

binds to *S.aureus* (Liu *et al.*, 2005). The lectin pathway of activating complement will be discussed in more detail in Section 1.8.1.

1.5. Mannose-binding lectin

Mannose-binding lectin (MBL – also known as mannan-binding lectin, mannose-binding protein and mannan-binding protein) is an acute phase serum protein of hepatic origin (Thiel *et al.*, 1992; Ezekowitz *et al.*, 1988; Taylor *et al.*, 1989; Sastry *et al.*, 1989). At present MBL has been described in a wide range of vertebrates (Kawakami *et al.*, 1984) including all mammals and birds which have been studied (Holmskov *et al.*, 1994; Turner *et al.*, 1996). Related proteins have also been described in an invertebrate species (Sekine *et al.*, 2001). Mannan-binding lectin (MBL) was first isolated from rabbit liver (Kawasaki *et al.*, 1978) by its strong binding to yeast mannan and subsequently found in the serum and liver of the rabbit (Kozutsumi *et al.*, 1980), rat (Mizuno *et al.,* 1981; Townsend and Stahl, 1981; Oka *et al.*, 1985), human (Kawasaki *et al.*, 1983), bovine (Kawasaki *et al.*, 1985), chicken (Oka *et al.*, 1985), mouse (Holt *et al.*, 1994), and pig (Storgaard *et al.*, 1996). MBL occurs in two distinct forms in rodents and other animals, including the rhesus monkey (Mogues *et al.*, 1996). In rodents the MBL-A variant has been considered to be the serum form, whereas MBL-C has been called the liver form, but it has recently been found that both forms appear in serum (Hansen *et al.* 2000). However, only one form has been found in the chicken and man. Human MBL is synthesised from a single functional gene on chromosome 10, named MBL2 (Sastry *et al.*, 1989). Mannose-binding lectin is an acute phase serum protein synthesised by hepatocytes (Ezekowitz *et al.,* 1988; Taylor *et al.*, 1989; Sastry *et al.*, 1989) which extravasate during inflammatory processes. MBL has been found in body fluids, including nasopharyngeal secretions, middle ear effusions, ascites, urine, CSF, amniotic fluid and synovial fluid (Garred *et al.*, 1993; Terai *et al.*, 1993).

In unstimulated human tissue e.g. the small intestine, a limited extra-hepatic expression of the MBL2 gene was observed (less than 6% of the hepatic

expression) (Seyfarth *et al.*, 2002). Another study revealed that extra-hepatic transcription of human MBL2 is taking place, especially in small intestine and testis tissue where the mRNA level comprised about 1% and 0.2%, respectively, of that seen in liver (Seyfarth *et al.*, 2005). More recently, significant intracellular expression and minor surface expression of MBL in adherent human monocytes and monocytes-derived dendritic cells has been described (Downing *et al.*, 2003). Another study found that differentiated monocytes of the THP-1 cell line showed low, but elevated levels of MBL2 mRNA transcribed from both promoters as compared to undifferentiated THP-1 cells, when both populations were incubated with LPS. No MBL2 mRNA was detected in human mononuclear cells isolated from blood irrespective of genotype, but very low levels of human MBL2 mRNA were found in cells from cord blood, suggesting that fetal haematopoietic cells express MBL (Seyfarth *et al.*, 2005).

As a member of the collectin family of proteins, containing collagenous and lectin domains, MBL is believed to be an important constituent of the innate immune system. It is thought to be most important during the "window of vulnerability" in infants (about 6 to 18 months) i.e. after the decay of maternal antibody and prior to the maturation of the child's own immune repertoire (Super *et al.*, 1989). Indeed, MBL levels have been shown to be higher in children aged from birth to 6 years than in older children and adults (Aittoniemi *et al.*, 1996). MBL has also been found in amniotic fluid suggesting that it may play a role in clearance of pathogens from the amniotic cavity (Malhotra *et al.*, 1994).

MBL is able to bind to a range of sugars which are present in particular conformations on the surface of microbial but not human cells or proteins (Weis and Drickamer, 1994; Sheriff *et al.*, 1994). On binding to its targets, MBL can activate complement (Ikeda *et al.*, 1987; Ohta *et al.*, 1990; Matsushita *et al.*, 1992) via mannose-binding lectin associated serine proteases (MASP-1, MASP-2 and MASP-3) (Ji *et al.*, 1993; Takayama *et al.*, 1994; Matsushita and Fujita, 1992; Thiel *et al.*, 1997; Dahl *et al.*, 2001) (Figure 1-1). This leads to either phagocytosis, or direct lysis of an invading pathogen (Kawakami *et al.*, 1982; Kawasaki *et al.*, 1989). In addition, MBL may be able to act as a direct opsonin (Kuhlman *et al.*, 1989;

Polotsky *et al.*, 1997; Hartshorn *et al.*, 1997), thereby enhancing phagocytosis (Levitz *et al.*, 1993; Tenner *et al.*, 1995).

1.6. MBL structure

1.6.1. Primary structure

MBL consists of polypeptides of approximately 32 kDa each, which are assembled into trimers of 96 kDa, which themselves combine to form higher order structures based on 2 – 6 such 3 chain subunits (Figure 1-3).

Each polypeptide contains an N-terminal domain of 21 amino acids, a collagen-like domain, a neck region and a C-terminal carbohydrate recognition domain (CRD) (Malhotra *et al.*, 1992).

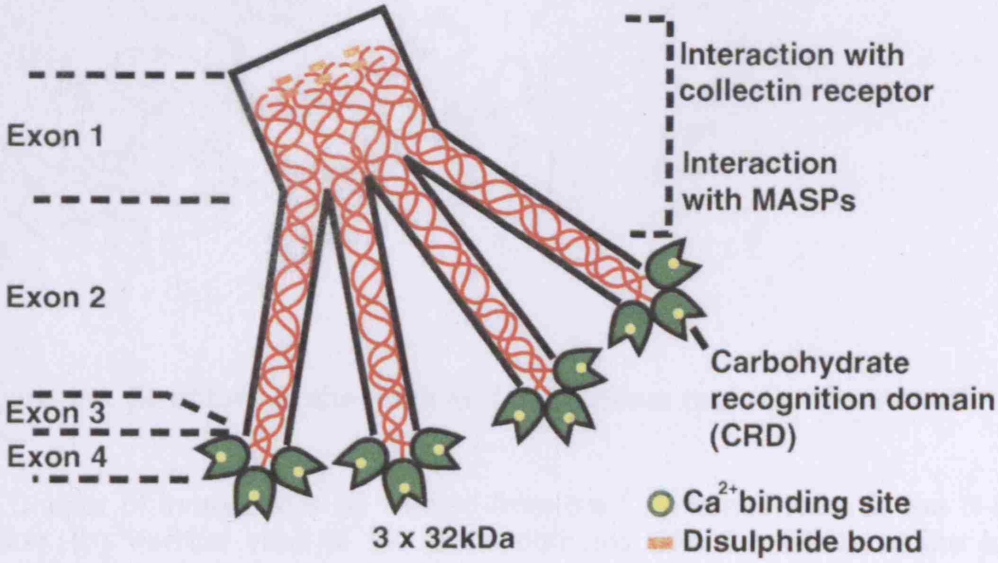


Figure 1-3. Mannose-binding lectin.

Representation of a tetrameric MBL molecule. The N-terminal region can be seen at the top of the diagram with the C-terminus at the bottom. The CRDs can be seen containing the Ca2+ binding sites. The corresponding exons encoding for each region are indicated on the left hand side. Taken from Turner and Hamvas (2000).

The N-terminal region of 21 amino acids, which is cysteine rich and probably provides stability within the subunits, is followed by the collagenous region consisting of 19 Gly-Xaa-Yaa repeats with a single interruption of this pattern (at

repeat 8) where the sequence is Gly-Gln-Gly. This may account for the divergence of the subunits (Taylor *et al.*, 1989; Sastry *et al.*, 1989). This collagenous region is connected to a C-terminal region of 115 amino acids by a "neck" region of 25 amino acids (Weis and Drickamer, 1994; Sheriff *et al.*, 1994) (Figure 1-4). Each subunit C-terminal region has three CRDs, one at the end of each polypeptide of the triple helix and each CRD contains a Ca^{2+} binding site, which is believed to be of critical importance for sugar binding.

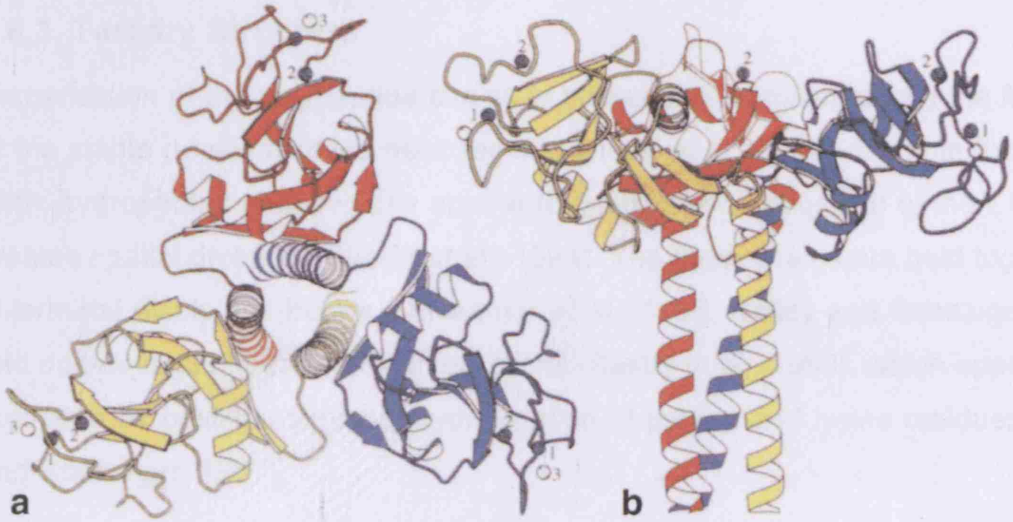


Figure 1-4. Structure of the neck and C-terminus regions of a subunit of rat MBL.

(a) Cluster of three CRDs as viewed from the C-terminus towards the N-terminal region. (b) Vertical view of the lectin domains with the CRDs at the top. The carbohydrate binding sites involved in sugar interactions are indicted by the calcium ions numbered 2.Taken from Weis and Drickamer (1994).

1.6.2. MBL sugar binding specificity

The sugar binding specificity of MBL is determined by the co-ordination bonds formed between the protein and the 3- and 4-hydroxyl groups of various sugars such as mannose, *N*-acetyl glucosamine, fucose and glucose. In contrast, MBL does not bind to proteins that do not have this carbon ring structure such as galactose and sucrose. The discrimination between foreign mannose-type sugars and host oligosaccharides is in part due to the spatial orientation of the CRDs

which are 45 Å apart for human MBL (Chang *et al.*, 1994; Sheriff *et al.*, 1994). In contrast to the repeating saccharides of microbes, mammalian sugars are rarely presented in such close proximity as to interact with a group of three CRDs (Weis and Drickamer, 1994). Though mammalian sugars may be able to interact with a single CRD, the affinity is very low. Simultaneous binding to multiple CRDs, however, as occurs with microbes, is thought to provide enough avidity for functional interactions. The sugar binding interactions of MBL differ depending upon the monosaccharide.

1.6.3. Tertiary Structure

Trimerisation of the polypeptide chains is believed to be initiated with the formation of the stable α-helical coiled neck region (Sheriff *et al.*, 1994). Once in the trimeric form, hydrophobic neck regions appear to inhibit close apposition of the CRDs and creates spatial diversity (Sheriff *et al.*, 1994). The three chains are held together by N-terminal disulphide bonds (Drickamer *et al.*, 1986; Colley and Baenziger, 1987) and non-covalent hydrophobic interactions (Sastry *et al.*, 1989), which appear to be mediated by post-translational hydroxylation of proline and lysine residues (Colley and Baenziger, 1987).

1.6.4. Quaternary structure

The quaternary structure of MBL is still a matter of some debate. The subunits of 3x32 kDa appear to combine to give oligomers ranging from dimers to hexamers as observed by electron microscopy (Lu *et al.*, 1990), although the mechanism of this association is still unknown. Various relative amounts of these oligomeric forms have been reported, with a predominance of hexamers (Wong H and R.B.Sim, 1997), of dimers and trimers (Lipscombe *et al.*, 1995), or of trimers and tetramers (Lu *et al.*, 1990; Teillet *et al.*, 2005). An alternative analysis using gel filtration indicated that the majority exist as trimers, tetramers and pentamers (Yokota *et al.*, 1995). Another study combining gel filtration and sucrose gradient centrifugation suggested that trimers were the most abundant form (Colley *et al.*, 1988). Electron microscopy has also shown these subunits to be arranged in a bouquet like structure, similar to that seen for C1q (Lu *et al.*, 1990).

1.7. MBL Genetics

1.7.1. The MBL gene

Human MBL is encoded by a single gene located on chromosome 10 at 10q21 and there is also a pseudogene located on the same chromosome (Guo *et al.*, 1998). The expressed functional MBL gene (MBL-2) consists of four exons and an upstream promoter region (see Figure 1-5). Each of the exons corresponds to a particular region of the protein: the first exon encodes the N-terminal region and the start of the collagenous region, exon 2 encodes the remainder of the collagenous region, exon 3 encodes the neck region of the molecule and exon 4 encodes the carbohydrate recognition domain found at the C-terminus. The gene has been reported to be upregulated in vitro by interleukin-6 (IL-6), dexamethasone and heat shock protein and downregulated by IL-1 (Arai *et al.*, 1993). More recently, growth hormone has been found to increase MBL *in vivo* in healthy adults and those with growth hormone deficiency (Hansen *et al.*, 2001). In addition, MBL levels were found to be lower in patients with growth hormone deficiency compared to normal controls and higher in patients with acromegaly, in whom MBL was reduced following treatment with the growth hormone receptor antagonist pegvisomant (Hansen *et al.,* 2001). Another study demonstrated that growth hormone, IL-6, T3 and T4 significantly increases MBL synthesis in a dose-dependent manner, while hydrocortisone, insulin and IGF-1 had no effect (Sørensen *et al.,* 2006).

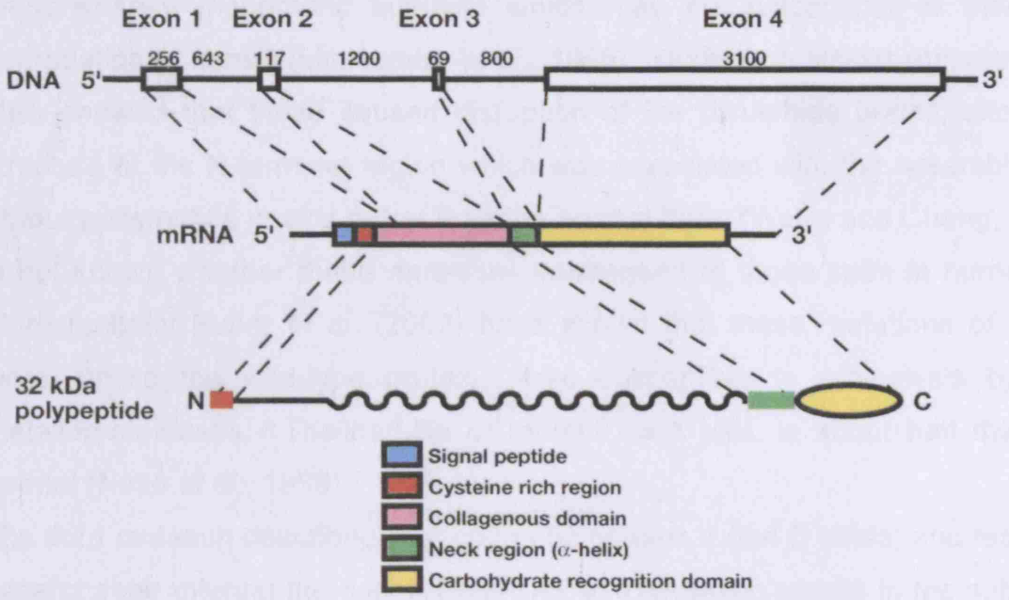


Figure 1-5. The human MBL gene with its corresponding mRNA transcript and protein.

The lengths of the various introns and exons are shown in base pairs. The dashed lines above the mRNA indicate which exon encodes for which part of the transcript and those below indicate which protein domains are encoded for by which part of the mRNA. Reproduced from Turner (2002).

1.7.2. MBL Structural gene mutations

Three structural gene mutations have been described in exon 1 of the MBL gene and each results in decreased levels of MBL. To date, no mutations have been described in exons 2, 3 or 4. The first of the mutations to be described is in codon 54 (denoted the B allele – with A being the wild type allele) where a base substitution results in the codon changing from the wild type GGC to GAC (Sumiya *et al.*, 1991). In the protein this mutation results in the replacement of a glycine by aspartic acid. Similarly a mutation in codon 57 (known as the C allele) alters the codon from GGA to GAA, and results in a glutamic acid residue being present instead of a glycine (Lipscombe *et al.*, 1992). Both of these substitutions are thought to cause low MBL levels by impairing polymerisation. Compared to the relatively compact structure of glycine, the bulky side chains of glutamic acid and aspartic acid probably distort the collagenous helix and impair the assembly of higher order multimers. This is believed to result in the hepatic secretion of

predominantly monomeric subunits which may be susceptible to more rapid degradation in serum (Lipscombe *et al.*, 1995). Studies on similar mutations in rat MBL showed that these caused disruption of the disulphide bonds, altering the structure of the N-terminal region which was associated with the assembly of two or four polypeptide chains rather than the normal three (Wallis and Cheng, 1999). It is not known whether these mutations correspond to those seen in human MBL. More recently Butler *et al.* (2002) have shown that these mutations of rat MBL were, unlike the wild-type protein, more susceptible to proteolysis by matrix metalloproteinases. The half-life of mutant mice MBL is about half that of the normal (Naito *et al.*, 1999).

The third mutation described is in codon 52 of exon 1 (the D allele) and results in a base change altering the codon from CAT to TAT which results in the substitution of a cysteine for an arginine and appears to have a less severe effect on circulating MBL levels (Madsen *et al.*, 1994). The disruption of even a single MBL subunit is likely to destabilise an MBL-MASP complex. Because MASP-2 in complex with MBL autoactivates and can trigger complement activation, it is more probable that MASP-2 activation itself is defective in complexes with mutant MBL (Chen and Wallis, 2001).

1.7.3. MBL Promoter polymorphisms

In addition to the structural gene mutations in exon 1 there are several polymorphisms in the upstream promoter region of the MBL gene, which can have a modulatory effect on the basal serum MBL level. There are three described promoter polymorphic sites: H / L (at –550), X / Y (-221) and P / Q (+4) (Figure 1-6), which occur in one of the following linkage groups: HYP, LYQ, LYP or LXP; superimposing very high, high, medium and low protein expression levels onto the exon 1 genotype respectively (Madsen *et al.*, 1995).

There is also linkage disequilibrium between the structural and promoter mutations such that the B allele is always linked to the LYP promoter genotype, the C allele is associated with the LYQ promoter genotype and the D allele is linked to the HYP promoter genotype. More recently, a Brazilian study of 250 individuals has claimed to find an individual of European origin with the LYPD allele, (Boldt and Petzl-

Erler, 2002) and this finding had been confirmed by another study which found LYD allele in 1.1% of all alleles in Eastern Europeans (Skalnikova *et al.*, 2004).

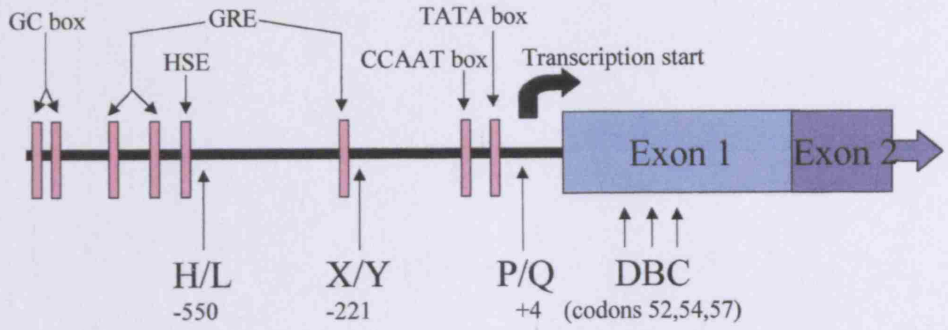


Figure 1- 6. Exon 1 and the promoter region of MBL.

The polymorphic sites are indicated at the bottom of the diagram. Above this schematic representation various gene regulatory elements are shown, including a heat shock element (HSE) and a glucocorticoid-responsive element (GRE). Reproduced from Turner (2000).

1.7.4. Worldwide distribution of MBL alleles

One of the most intriguing aspects of MBL genetics is the high incidence of mutations that have been shown to occur in various population groups. The frequency of mutations observed have been shown to range from 0.50 in both a Mapuche population from southern Argentina (Madsen *et al.*, 1998) down to zero in the Warlpiri indigenous Australian tribe (Turner *et al.*, 2000).

The B allele occurs at particularly high rates in many Eurasian populations, whereas the C allele is mainly found in Afro-Caribbeans and individuals of African ancestry. These interesting geographical distributions are highlighted in Figure 1-7.

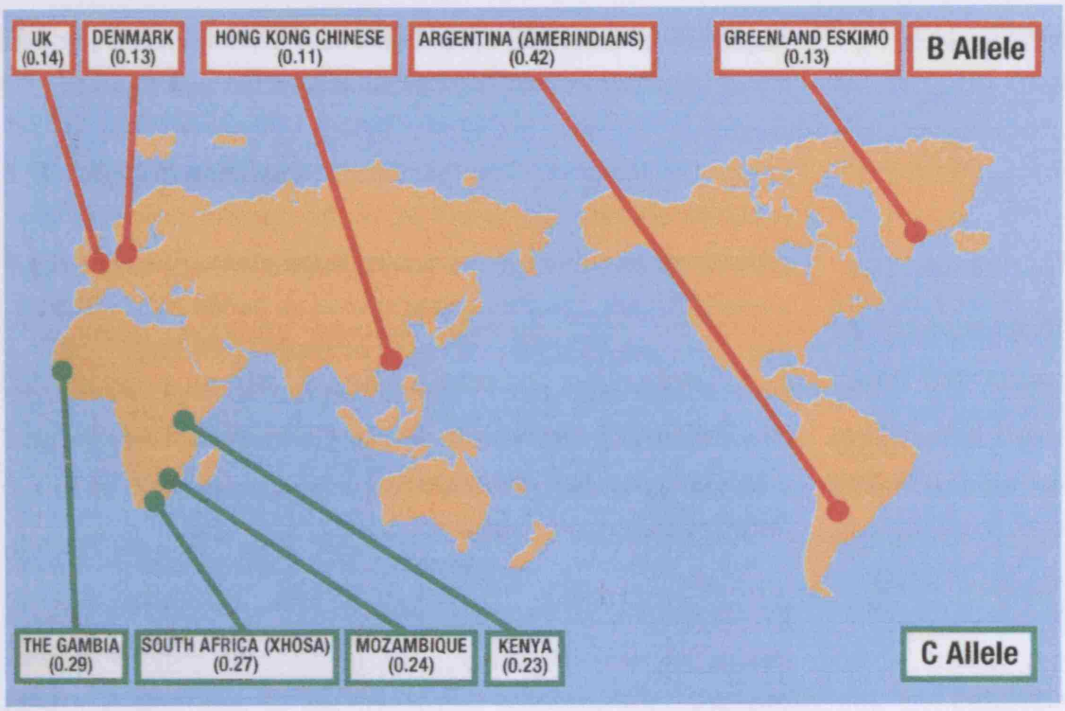


Figure 1-7. Some populations with high frequencies of the B and C alleles.

The clustering of populations with a high frequency of the C allele in Southern and sub-Saharan Africa can be seen compared to the more widespread incidence of the B allele. Interestingly, the highest frequency of the B allele (0.46) occurs in South America. No population has yet been described with a frequency for the D allele of higher than 0.07. Reproduced from Turner (2000).

These results have suggested that, at least in certain parts of the world, there must be a selection pressure favouring lower MBL levels. One hypothesis which was proposed is based on the premise that MBL increases C3b opsonisation of some intracellular bacteria and parasites and thereby facilitates uptake by phagocytic macrophages (Garred *et al.*, 1997a; Kahn *et al.*, 1996 Hoppe *et al.*, 1997). Thus a lower MBL basal serum level may confer some protection against these pathogens. Interestingly, an absence of structural MBL gene mutations was found in indigenous Australians and this might in part explain the impact of tuberculosis on this population when it was introduced by European settlers in the 19th and 20th centuries (Turner *et al.*, 2000).

An alternative hypothesis to explain the high frequency of mutant MBL alleles is related to the ability of MBL to activate complement. A deficiency of the lectin may reduce complement activation and may be beneficial by modulating inflammation

and subsequent host tissue damage (Lipscombe *et al.*, 1992). These two hypotheses are not necessarily mutually exclusive.

1.8. MBL function

1.8.1. The lectin pathway of complement activation

The lectin pathway involves carbohydrate recognition by pattern-recognition receptors, such as mannose-binding lectin and ficolins, and the subsequent activation of associated serine proteases (MASPs). MBL and ficolins have been found to be associated with MASP-1, MASP-2, MASP-3 (which is generated by alternative splicing of MASP-1) (Dahl *et al*, 2001) and a non-protease, small MBL-associated protein (sMAP or Map19; a truncated form of MASP-2) (Stover *et al.*, 1999).

MASP-2 appears to be the most important of the proteases by catalysing the cleavage of C4 to C4a and C4b, and C2 to C2a and C2b, thereby resulting in the formation of the C3 convertase C4b2a which cleaves C3 into C3a and C3b (Thiel *et al.*, 1997). The C3b fragments can then become attached to the cell surface in close vicinity to C4b2a or to C4b2a itself and creating the C4b2a $(3b)_n$ complex, which is the C5 convertase of the classical pathway. Initiation of the terminal pathway can then occur in the same way as for the classical and alternative pathways resulting in formation of the membrane attack complex (Fig.1-1).

This result has been confirmed by the functional analysis of recombinant MASP-2 (Vorup-Jensen *et al*, 2000; Wallis and Dodd, 2000; Chen and Wallis, 2001; Thielens *et al.,* 2001). MBL isolated from serum is apparently only associated with MASP-1 and MASP-2 and not C1r or C1s (Thiel and Jensenius, 1997). It has been reported that MASP-1 is able to cleave C3 directly (Dahl *et al.*2001; Matsushita and Fujita, 1995; Rossi *et al.* 2001), which can result in the activation of the alternative pathway (Matsushita and Fujita, 1995). However, other research suggested that it cleaves C3 (H_2O) (dead C3), but not C3, at a significant rate (Hajela *et al.* 2002). MASP-1 does not appear to have a role in activating MASP-2, and has very limited or negligible activity towards other complement proteins. In a survey of potential synthetic and natural substrates and inhibitors, MASP-1 has a thrombin-like

activity: it cleaves fibrinogen and activates plasma transglutaminase (factor XIII) (Wong and Sim, 1999).

The functions of MASP-3 and MAp19 (the truncated form of MASP-2) are currently unknown. sMAP may prevent inadvertent activation of the MBL-MASP complex before microbial infection or suppress overactivation of the lectin pathway once activated (Iwaki *et al.*, 2006). MASP-3 is also a competitor of MASP-2 in binding to MBL and down-regulates the C4 and C2 cleavage activity of MASP-2 (Dahl *et al.*, 2001). Although the interaction between sMAP and MASP-3 has not been investigated, it is possible that they are able to down-regulate activation of the lectin pathway cooperatively (Iwaki *et al.*, 2006).

Like the other pathways of complement activation, the lectin pathway is inhibited and regulated by other factors present in serum. Both C1-inhibitor and α2-macroglobulin have been reported to inhibit MBL / MASP activation of the lectin pathway in *vitro*, although the exact mechanisms underlying this inhibition have yet to be elucidated (Petersen *et al.*, 2000; Wong *et al.*, 1999; Matsushita and Fujita, 1996; Terai *et al.*, 1995; Gulati *et al.*, 2002). A recent study investigated the mechanism of formation of complexes between α2M and MBL and concluded that they form by direct binding of oligomannose glycans Man_{5-7} occupying Asn-846 on α2M to the lectin domains of MBL (Arnold *et al.*, 2006). The oligomannose glycans are accessible for lectin binding on both active α2M (thiol ester intact) and protease-cleaved α2M.

1.8.2. Opsonisation

In addition to generating opsonic C3b fragments there is some evidence to suggest that MBL can act directly as an opsonin to enhance phagocytosis (Kuhlman *et al.*, 1989; Polotsky *et al.*, 1997; Hartshorn *et al.*, 1993). Native and recombinant human MBL were found to bind directly to wild-type virulent *Salmonella montevideo* expressing a mannose-rich O-polysaccharide and resulted in the attachment, uptake, and killing of MBL-coated bacteria by phagocytes in the absence of serum (Kuhlman, M. *et al.* 1989). Preincubation of *M.avium* with recombinant MBL resulted in an increase in uptake by human neutrophils (Polotsky *et al.*, 1997). The phagocytosis of *Cryptococcus neoformans* has been described as being significantly enhanced by recombinant MBL (Levitz *et al.*, 1993). It seems that MBL

can bind to autologous apoptotic cells, presumably promoting the clearance of cellular debris (Ogden *et al.*, 2001).

The possible receptors for MBL mediated phagocytosis are still a matter of some dispute and several possible receptors have been implicated including cC1qR / calreticulin (Malhotra *et al.*, 1994, Ogden *et al.*, 2001), $C1qR_p$ / CD93 (Steinberger *et al.*, 2002; Tenner *et al.*, 1995) and complement receptor 1 / CD35 (Ghiran *et al.*, 2000). Two recent studies have suggested that scavenger receptor A might have a role as an MBL receptor after finding that MBL augments the SR-A mediated uptake of lipid A by Kupffer cells (Ono *et al.*, 2006; Konishi *et al.*, 2006).

1.8.3. Cytokine activation

MBL has also been shown to be important in the regulation of cytokine release from phagocytic cells in experimental models of infection.

In vitro the production of TNF-α by monocytes has been shown to increase in the presence of *Cryptococcus neoformans* mannoprotein when recombinant MBL was added (Chaka *et al.*, 1997). This increase in cytokine production by monocytes was also observed in response to *Candida albicans* when this organism was incubated with rabbit MBL (Ghezzi *et al.*, 1998). A more recent study by Jack *et al.* (2001) demonstrated that production of the pro- inflammatory cytokines IL-1β, IL-6 and TNF-α from human monocytes stimulated with meningococci, initially increased when MBL was added, but decreased at very high concentrations (>6 μg /ml), suggesting that MBL plays an important dose dependent role in the regulation of the inflammatory response.

However, these findings were apparently at variance with an earlier *in vitro* study of TNF-α production by monocytes stimulated with rhamnose glucose polymers (RGPs) from streptococci where addition of human MBL was found to decrease cytokine production (Soell *et al.*, 1995). These authors suggested that in their experimental system the binding site of the RGPs for MBL was able to compete with the CD14 binding site on the monocyte surface.

1.9. MBL Disease Associations

1.9.1. General susceptibility

The first reported case of human MBL deficiency described was probably that of Miller *et al.* (1968) who described their observations of an infant with recurrent infections, failure to thrive and diarrhoea attributed to an idiopathic opsonic defect. Later investigations suggested that there was a defect of opsonisation associated with frequent unexplained infections (Soothill and Harvey, 1976), chronic diarrhoea (Candy *et al.*, 1980) and *Otitis media* (Richardson *et al.*, 1983), and that this defect was present in 5 – 7 % of the population (Soothill and Harvey, 1976). In 1989, a study of individuals with a failure to opsonise Baker's yeast (*Saccharomyces cerevisiae*) found an association between this common opsonic defect and MBL deficiency (Super *et al.*, 1989). Furthermore, it was shown that the addition of MBL to serum with low C3b binding activity led to an increased deposition of C3b, C3bi, Factor B and C4 (Super *et al.*, 1989).

Polymorphisms in the MBL gene leading to decreased serum MBL levels (see Section 1.7) have since been shown to be important in recurrent infections (Garred *et al.*, 1995; Summerfield *et al.*, 1997; Kakkanaiah *et al.*, 1998). MBL deficiency has also been associated with increased episodes of acute respiratory tract infections (Koch *et al.*, 2001), but not acute otitis media, in children from Greenland (Homoe *et al.*, 1999).

In a study of adults with non-HIV related immunodeficiencies it was found that this group had a higher proportion of individuals homozygous for MBL structural (exon 1) gene mutations than healthy controls (Garred *et al.*, 1995). A later study of consecutive paediatric admissions with and without infections showed that there was a significant increase in those heterozygous or homozygous for MBL structural gene mutations in the group with infections compared with children admitted to hospital without evidence of infection (Summerfield *et al.*, 1997). Kakkanaiah *et al.* (1998) have shown that the proportion of individuals with serum MBL concentrations of less than 5 ng/µl are significantly increased in both children and

adults with recurrent infections compared to age-matched healthy controls. Recently, a study of individuals with common variable immunodeficiency (CVID) found no differences in the proportions of individuals heterozygous or homozygous for structural gene mutations in the patients compared to controls. However, this study did find that the age of onset of disease was significantly lower in those heterozygous or homozygous for structural gene mutations, compared to wild-type individuals (Mullighan *et al.*, 2000).

Several studies have been carried out to investigate whether MBL deficiency is important in susceptibility to infection in malignancy. Peterslund *et al.* (2001) measured MBL serum concentrations in adult leukemic patients prior to chemotherapy and found an association between low concentrations and severe infections following treatment. Similarly, in a prospective study of children with malignancies those with low serum MBL concentrations and / or structural exon1 gene mutations (A/O plus O/O) prior to chemotherapy were more likely to have longer febrile neutropenic episodes than those with normal / high MBL levels and no such mutations (A/A) (Neth *et al.*, 2001).

Subsequently, another study reported that MBL gene polymorphisms were associated with major infections following allogeneic haemapoietic stem cell transplantation, with invasive bacterial, viral, and fungal infection occurring significantly more frequently among those with variant MBL alleles. Both the donor and recipient MBL status were found to be important with the association between MBL2 mutations and major infections more statistically significant for donor haplotype. The presence of the HYA promoter haplotype in either donor or recipient appeared to protect against infection (Mullighan *et al.*, 2002).

However, two later studies have failed to confirm the above findings. Both examined adults with malignancies. Bergmann *et al.* (2003) studied 80 patients with acute myeloid leukaemia and found no differences in frequency, severity or duration of fever in MBL replete or deficient patients. Kilpatrick *et al.* (2003) studied 128 patients with more than half diagnosed with lymphoma or AML and in whom two-thirds were receiving conditioning prior to bone marrow transplantation. A major influence of MBL levels on rates of severity of infection was not detected, but a modest influence of MBL concentrations <100 ng/ml on susceptibility to infection was confirmed (Kilpatrick *et al.*, 2003). Studies by Aittoniemi *et* al., (1999) (28

patients with chronic lymphocytic leukaemia) and Tacx *et al* (2003) (177 patients with new onset fever admitted to an internal medicine department) with few details did not observe any effect of MBL on infections. Two recent studies failed to demonstrate a protective effect of wild-type MBL2 genotype on chemotherapy-related infections in patients with multiple myeloma (Mølle *et al.*, 2006) and in paediatric oncology patients with neutropenia (Frakking *et al.*, 2006).

It has also been proposed that MBL could act as a potential modifier of disease in other immunodeficiencies. Evidence in support of this comes from a study of chronic granulomatous disease (CGD) in which a significant proportion of individuals with rheumatologic and autoimmune disorders were found to have MBL structural gene mutations (Foster *et al.*, 1998). However, no association has been shown between MBL deficiency and increased susceptibility to infection in patients with IgA deficiency (Aittoniemi *et al.*, 1999).
Another group of patients where the immune system may be suppressed is critically ill patients admitted to ICUs. Systemic inflammatory response syndrome (SIRS) may render the patients partly immunocompromised and may in some cases progress to sepsis and septic shock. The association between MBL deficiency and SIRS will be reviewed in Chapter 5.

1.9.2. MBL and viruses

The vast majority of studies on MBL and viruses have been performed using HIV, influenza and hepatitis and these are reviewed below together with a single herpes simplex virus-related study and severe acute respiratory syndrome coronavirus study.

1.9.2.1. Human Immunodeficiency Virus (HIV)

Many studies have sought to find associations between MBL deficiency and infection with Human Immunodeficiency Virus (HIV). Interest in associations between MBL and HIV began with the study of Ezekowitz *et al.* (1989) which showed that purified MBL was able to bind to HIV-infected cell lines and directly inhibit HIV infection of lymphoblasts. MBL binds to and activates complement on gp120 (Haurum *et al.*, 1993), which is rich in mannose residues and is critical for

interactions of the virus with the cell-surface marker, CD4, on the surface of the T cells targeted by virus.

MBL binds to both CCR5 and CXCR4 tropic primary isolates of whole virus (Saifuddin *et al.*, 2000). Recent data indicate that MBL can opsonise HIV but does not influence neutralisation at the levels at which it is normally present in serum (Ying *et al.*, 2004). However, binding and opsonisation of HIV by MBL may alter virus trafficking and viral-antigen presentation during HIV infection. MBL may influence uptake by dendritic cells, which express a cell-surface lectin called "dendritic cell-specific intracellular adhesion molecule3 (ICAM-3)-grabbing non-integrin" (DC-SIGN). Preincubation of HIV strains with MBL prevents DC-SIGN-mediated trans infection of T cells and indicates that in vitro, MBL may inhibit DC-SIGN-mediated uptake and spread of HIV (Ying *et al.*, 2004).

Despite the impressive in vitro data, epidemiological studies have been less conclusive. There is broad agreement that MBL deficiency is associated with a greater susceptibility to HIV acquisition. MBL deficiency increased the acquisition of HIV infection by between three-and eight-fold (Garred *et al.*, 1997; Nielsen *et al.*, 1995; Prohászka *et al.*, 1997) and increased the risk of vertical transmission from infected mothers to other offsprings (Boniotto *et al.*, 2000). However, the minority of studies have failed to demonstrate a role of MBL in HIV infection (Malik *et al.*, 2003; McBride *et al.*, 1998; Senaldi *et al.*, 1995).

There is less clarity with regard to the role of MBL in HIV disease progression. Garred *et al.* (1997) demonstrated that men with MBL variant alleles had a shorter survival time following the onset of AIDS than did patients with wild-type MBL alleles. However, in a well characterised cohort of homosexual men, variant MBL alleles had an insignificant effect on survival following the diagnosis of AIDS (Maas *et al.*, 1998). In this latter study, there appeared to be a protective effect of MBL variant alleles with a delay in the development of AIDS from the time of HIV seroconversion. Patients with MBL variant alleles had lower CD4 counts at the time of developing AIDS, indicating that MBL deficiency may influence the onset of AIDS for any given CD4 count. Furthermore, MBL mutations appeared to protect against the development of Kaposi sarcoma, a finding that was difficult to explain (Maas *et al.*, 1998). Prohaszka *et al.* (1997) found that MBL levels were lower in asymptomatic HIV-positive individuals when compared with HIV-negative controls.

However, the protective effect of MBL was lost in patients with AIDS diagnosis; patients with high MBL levels had significantly lower numbers of CD4 cells. One study has attempted to relate MBL status and HIV-infected long-term non-progressors (Hundt *et al.*, 2000). Another study demonstrated that children with rapidly progressing disease are more likely to have MBL variant alleles (B) than slower progressors (Amoroso *et al.*, 1999).

A study of 72 Zambian AIDS patients found that individuals homozygous for MBL structural gene mutations were at increased risk of developing cryptosporidiosis (Kelly *et al.*, 2000). Furthermore, MBL was present in small intestinal fluid and could bind to *Cryptosporidium parvum* sporozoites and activate complement (Kelly *et al.*, 2000). The presence of the variant MBL B allele was associated with higher plasma viral load levels in 145 HIV-1 infected Brazilian patients (Vallinoto *et al.*, 2006). It had to be stated that the results of these studies are limited by the numbers of patients enrolled. None of these studies are sufficiently powered to elucidate meaningful differences between groups. It would appear that MBL is implicated as a determinant of HIV acquisition and progression but its involvement has not yet been clearly elucidated.

1.9.2.2. Influenza

Interest in MBL and influenza was sparked by early research showing that MBL could bind to the neuraminidase of influenza A virus (Malhotra *et al.*, 1994). In 1993, Hartshorn and colleagues had shown that MBL could inhibit the haemagluttinin activity of several strains of Influenza A and enhance phagocytosis by its opsonisation properties. Anders *et al.* (1994) extended these results, demonstrating that guinea pig MBL had haemagluttination-inhibition and virus-neutralisation activities against Influenza A and B.

Interestingly, in a later study MBL was found to neutralise different strains of Influenza A depending on the amount and pattern of glycosylation of the virus (Reading *et al.*, 1997). Most recently the suggestion that MBL is able to neutralise Influenza A by opsonisation and complement activation has been confirmed and evidence has been presented that it may also directly neutralise the virus (Kase *et al.*, 1999).

1.9.2.3. Hepatitis

Higher than expected frequencies of the MBL D allele (codon 52) were associated with chronic hepatitis B infection in UK Caucasians, but not in Asians (Thomas *et al.*, 1996). However these findings were not supported by Höhler *et al.* (1998) who found no differences in the frequencies of the D allele or C allele when comparing German Caucasians with chronic hepatitis B to those with acute hepatitis B and controls. Similarly, in a Gambian study no association was found between MBL and hepatitis B (Bellamy *et al.*, 1998).

In contrast to the above findings various groups in Asia have described associations between MBL and various disease manifestations of hepatitis. Yuen *et al.* (1999) found that the B allele (codon 54) frequency was associated with cirrhosis and spontaneous bacterial peritonitis (but not hepatitis B-related cellular carcinoma) in Chinese patients with chronic hepatitis B infection, and more recently it has been shown that the frequency of this allele is significantly higher in nonsurvivors than survivors (Hakozaki *et al.*, 2002). In a study of Japanese patients with hepatitis C Matsushita *et al.* (1998) found a lower frequency of individuals homozygous for the B allele in interferon responders compared to nonresponders and controls, suggesting that MBL could usefully supplement interferon therapy in virus clearance. A later study of Japanese patients, which examined individuals homozygous or heterozygous for the LXPA or LYPB alleles, confirmed that they were less frequently observed in interferon responders than nonresponders or controls (Matsushita *et al.*, 1998). These findings are also supported by those of Sasaki *et al.* (2000) who looked at the B mutation in Japanese and found that the presence of this allele was more common in groups of patients with liver cirrhosis and chronic active hepatitis, a more severe form of the disease, again suggesting that low MBL may be associated with a poor prognosis in hepatitis C infection.

In 2005, Chong *et al.* reported that MBL genotypes correlating with low protein levels were associated with the occurrence of cirrhosis and also hepatocellular carcinoma in hepatitis B carriers (Chong *et al.*, 2005). They also demonstrated that MBL was able to bind to hepatitis B surface antigen. In another recent case-control study it was found that MBL genotypes correlating with high serum levels were

associated with recovery from infection (n=338), whereas those correlating with lower levels were associated with persistence of virus (n=189) (Thio *et al.*, 2005).

1.9.2.4. Other viruses

MBL genotype has been investigated in one study of patients with herpes simplex virus-associated Mollaret's meningitis, a rare complication of infection with this virus. In this study an increase in the incidence of the D allele was reported in the patient group compared with ethnically matched controls. However, the numbers in the patient group were very small (20), of whom 4 were found to have the D allele present) (Tang *et al.*, 2000). Further studies are required to evaluate the role of MBL in this disease.

The distribution of MBL gene polymorphisms was found to be significantly different between patients infected the coronavirus responsible for SARS (569) and in control subjects (1188) (Ip *et al.*, 2005). Serum levels of MBL were also significantly lower in patients with SARS than in control subjects. There was, however no association between MBL haplotype, which are associated with low or deficient serum levels of MBL, and mortality related to SARS. MBL can bind SARS-CoV in a dose- and calcium-dependent and mannan-inhibitable fashion in vitro (Ip *et al.*, 2005). A recent study demonstrated that MBL binds to viral particles bearing either Ebola (Zaire strain) or Marburg (Musoke strain) envelope glycoproteins (Ji *et al.*, 2005) and blocks viral DC-SIGN-mediated binding to cells. The complement neutralisation of both viruses was partially mediated through the lectin pathway (Ji *et al.*, 2005).

1.9.3. Bacteria

Many studies investigating the binding of MBL to various bacterial species have been carried out with one of the earliest of these investigating bacterial pathogens known to cause meningitis. In this investigation MBL was found to bind to some of the species studied but not to others, with the highest MBL binding being observed to *Salmonella montevideo* and various clinical isolates of *Listeria monocytogenes* (van Emmerick *et al.*, 1994). MBL has also been shown to bind to some bacterial pathogens isolated from immunocompromised children (see Figure 1.8), including most isolates of *Staphylococcus aureus* and beta –haemolytic group A streptococci

(Neth *et al.*, 2000). Devyatyarova-Johnson *et al.* (2000) showed that MBL was able to bind to some mutants of *Salmonella enterica* serovar *Typhimurium* and *Neisseria gonorrhoeae* and that this binding was dependent upon the lipopolysaccharide structures of these organisms. MBL binds significantly to peptidoglycan (PGN) via the N-acetyl Glucosamine moiety of Gram-positive bacteria (Nadesalingam *et al.*, 2006) and inhibits PGN-induced production of proinflammatory cytokines while enhancing the production of chemokines by macrophages.

MBL has also been found to bind to a range of obligate anaerobes capable of causing invasive disease in humans including *Bacteroides bifidium*, *Proprionibacterium acnes*, *Actinomyces israelii* and various species of fusobacteria (Townsend *et al.*, 2001). MBL binding to different bacterial structures will be reviewed in Chapter 3.

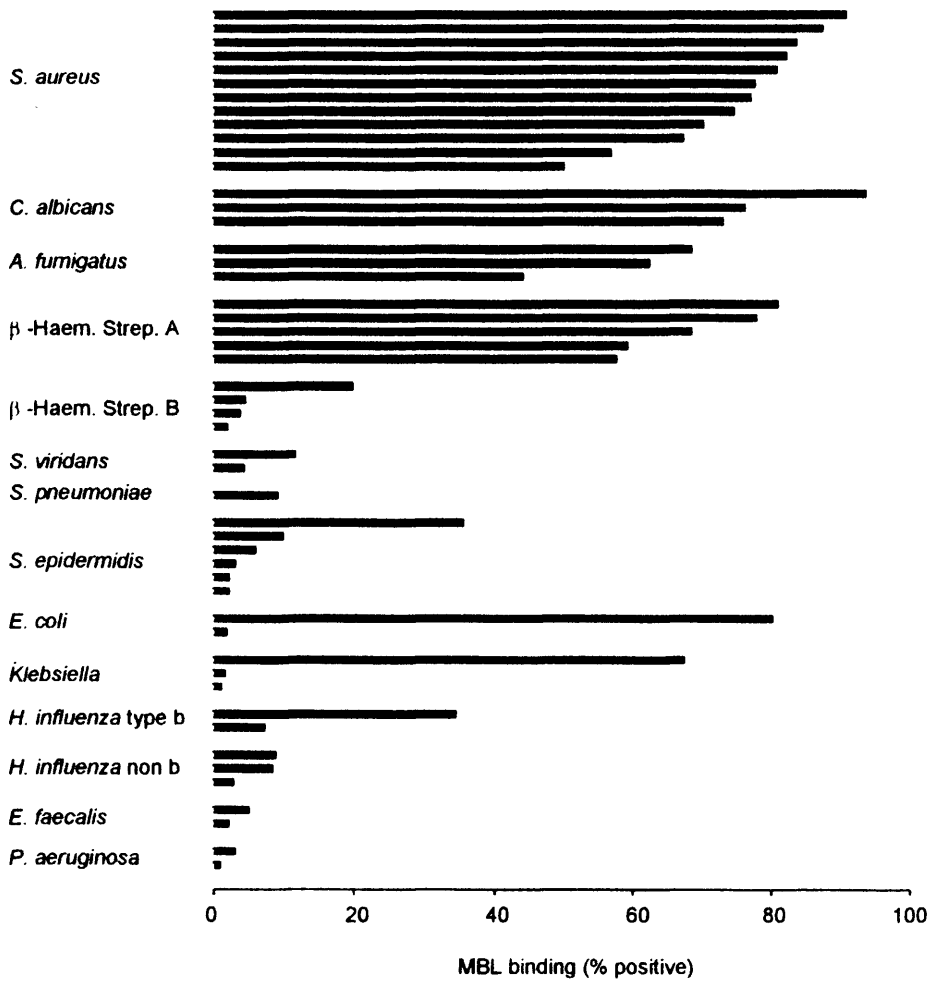


Figure 1- 8. The percentage of organisms positive for MBL binding of a range of clinical isolates.

The percentage positive were determined by flow cytometry. Taken from Neth *et al.* (2000).

1.9.3.1. Tuberculosis

Mycobacteria are intracellular parasites, which may gain access to cells after MBL-MASP complement activation deposits C3b onto the bacteria. In 1996, MBL was shown to opsonise and enhance phagocytosis of *Mycobacterium avium* (Polotsky *et al.*, 1996) providing evidence to support this theory. Further evidence came from a study of 36 Ethiopian patients with *Mycobacterium leprae* infection who were found to have significantly higher serum MBL levels compared to 26 healthy Ethiopians (Garred *et al.*, 1994).

The high frequency of B and C alleles in the tropics may confer protection against infection by intracellular parasites since the latter require coating by C3b opsonins for effective uptake by macrophages and MBL deficiency would significantly reduce the amounts of C3b deposited. Indeed the presence of the MBL B allele was implicated in protection against tuberculous meningitis in a South African population (Hoal-Van Helden *et al.*, 1999). Furthermore, Turner *et al.* (2001) have suggested that the lack of structural gene mutations in indigenous Australians may have contributed to the high susceptibility of these populations to tuberculosis when the disease was introduced from Europe in the 19th and 20th centuries.

Although MBL deficiency was, initially, not shown to be protective against pulmonary tuberculosis in the Gambia (Bellamy *et al.*, 1998) a subsequent study of this population found that the frequency of the C allele was lower among tuberculosis cases than in controls, but this was only of borderline significance (Bellamy, 2000). It thus remains unclear whether the presence of the C allele provides protection against intracellular invasion by *Mycobacterium tuberculosis*. In contrast, two studies found an increased frequency of functional mutant homozygote in patients with pulmonary tuberculosis compared to normal healthy subjects (Selvaraj *et al.*, 2006; Selvaraj *et al.*, 2004).

Garred and co-workers have also studied the influence of MBL levels on Sub-Saharan African HIV/AIDS and tuberculosis epidemics and found that HIV negative individuals with tuberculosis had higher MBL levels than Tanzanian controls or HIV positive individuals (Garred *et al.*, 1997). This indicated that high levels of MBL could be associated with an increased susceptibility to tuberculosis in immunocompetent individuals, supporting the theory that MBL may assist in opsonophagocytosis and uptake of intracellular bacteria. A more recent study found a significant decrease of genotypes carrying at least one O allele (XA/O, YA/O and O/O) in the HIV-TB group compared to HIV-non-TB patients or controls (Garcia-Laorden *et al.*, 2006). The same group did not find any differences in MBL frequencies between white Spanish HIV-negative TBC patients and controls.

1.9.3.2. Pneumococcal infection

Bacteraemia caused by the organism *Streptococcus pneumoniae* is a significant cause of morbidity and mortality in adults in the developed world. A study by van

Emmerik *et al.* (1994) using an isotope binding procedure showed that MBL was able to bind to ten clinical isolates from patients with streptococcal meningitis. In contrast, a later study based on flow cytometry found no evidence of binding of MBL to eleven laboratory strains or five clinical isolates of *S. pneumoniae* (Neth *et al.*, 2000). Two recent studies of the role of MBL deficiency in pneumococcal disease have also come to completely different conclusions. In one of these studies the MBL genotypes of 114 adult patients with pneumococcal bacteraemia did not differ from 250 healthy controls, nor were they associated with survival (Kronborg *et al.*, 2002). However, in the study by Roy *et al.* (2002) the frequency of individuals homozygous for MBL structural gene mutations was found to be significantly increased in patients (28/229) with invasive pneumococcal disease compared to controls (18/353). This initial sample was followed by a second confirmatory study where 10% (11/108) of patients were found to be homozygous for exon 1 mutations compared to 5% (36/679) of controls. A more recent study found a nonsignificant increased risk between the MBL structural variants (52, 54 and 57) and invasive pneumococcal disease (Moens *et al.*, 2006).

1.9.4. Yeasts and Fungi

MBL has long been suspected of playing a role in susceptibility to fungal infections. Indeed, the first cases of human MBL deficiency described were individuals whose serum failed to opsonise Baker's yeast (*Saccharomyces cerevisiae*) for phagocytosis by neutrophils (Miller *et al.*, 1968; Soothill and Harvey, 1976; Super *et al.*, 1989).

1.9.4.1. *Candida albicans* infection

MBL has been shown to bind to a range of clinical isolates of *Candida albicans* in vitro (Neth *et al.*, 2000) and studies in mice have shown that injecting *C. albicans* leads to an initial serum depletion of MBL, suggesting that in vivo MBL is binding to this yeast (Tabona *et al.*, 1995). Other studies also point to complex interactions between MBL and *C. albicans* infection. For example rabbit MBL was shown to increase in vitro production of TNF-α by monocytes in response to *Candida albicans* (Ghezzi *et al.*, 1998) and another study of bone marrow derived murine macrophages found that phagocytosis of *C. albicans* was inhibited by increasing

concentrations of rabbit MBL from 5 μg ml^{-1} to 50 μg ml^{-1} (Kitz *et al.*, 1992). MBL plays an important role in the first-line defence against *C.albicans* without the need for opsonophagocytosis by dendritic cells, in which a direct interaction of MBL with *C.albicans* results in agglutination and accelerated complement activation via the lectin pathway, leading to inhibition of growth (Ip *et al.*, 2004).

1.9.4.2. Cryptococcus neoformans infection

Cryptococcus neoformans is a yeast which can be a major opportunistic pathogen in immunocompromised patients. MBL binding to *C. neoformans* has been shown to occur with some unencapsulated strains, and the presence of capsule seems to decrease binding (Schelenz *et al.*, 1995; Neth *et al.*, 2000). In addition, the presence of recombinant MBL has been shown to increase binding to monocytes, neutrophils and macrophages and increase TNF-α production from monocytes (Levitz *et al.*, 1993; Chaka *et al.*, 1997).

1.9.4.3. Aspergillosis

Using flow cytometry, Neth *et al.* (2000) found that MBL could bind to several clinical strains of the fungi *Aspergillus fumigatus*. Recently, the presence of MBL mutations in 11 patients with chronic necrotising pulmonary aspergillosis was investigated and a higher frequency of MBL mutations were observed in these patients compared to ethnically matched controls (Crossdale *et al.*, 2001).

1.9.5. Parasitic diseases

It has been suggested that the high frequency of MBL mutations in some populations could have been sustained over time as a host defence mechanism against invasion of host cells by intracellular pathogens (Garred *et al.*, 1997; Kahn *et al.*, 1996; Hoppe *et al.*, 1997). Consequently several studies have now been carried out to investigate the role of MBL in parasitic disease.

1.9.5.1. Malaria

Malaria remains one of the most important parasitic diseases in the world and it is not unexpected that a possible role for MBL has been sought. In a prospective study of 200 Gabonese children (100 with severe malaria and 100 controls matched for age, sex and geographical area) the B and C alleles were found to be

increased in those with severe disease compared to those with mild malaria suggesting that MBL may play a role in disease progression (Luty *et al.*, 1998). However, the investigations of Bellamy *et al.* (1998), did not support these results and found no association between MBL and clinical malaria in a retrospective study of Gambian children (504 with severe malaria, 292 mild-malarial controls and 426 non-malarial age, sex and ethnically matched controls).

Despite these conflicting results it is of interest that MBL has recently been shown to bind to glycosylated parasite-derived proteins of *Plasmodium falciparum* infected cells (Klabunde *et al.*, 2002).

1.9.5.2. Leishmania

Leishmania are parasites which depend upon phagocytosis by macrophages for part of their life cycle and survival in the host, and thus have been proposed as one of the pathogens for which MBL levels may be modulatory. Low levels of the protein would protect against disease by reducing the amount of surface C3b deposition and subsequent activation by macrophages.

This theory is supported by the finding that in vitro MBL does bind to surface carbohydrates of *Leishmania* and also to the proteophosphoglycan secreted by amastigotes of *Leishmania mexicana*, resulting in complement activation (Green *et al.*, 1994; Peters *et al.*, 1997). This is thought to contribute to lesion development and heighten the severity of the disease. A study conducted on a related pathogen, *Leishmania chagasi*, found that median MBL levels were indeed higher in individuals with visceral leishmaniasis which is often fatal, than in healthy individuals who were either skin-test positive or negative for *Leishmania* (Santos *et al.*, 2001). Furthermore, patients with visceral leishmaniasis were more likely to be wild type for the exon 1 region of MBL and MBL serum levels could be directly positively correlated with the probability of developing this disease.

1.9.5.3. Other parasites

Trypanosoma cruzi is an intracellular parasite which is the causative agent of Chagas disease in humans. It has been shown that MBL is able to bind to surface glycoproteins of the *T. cruzi* amastigotes, which can only multiply intracellularly, suggesting that MBL could facilitate entry into mammalian cells in Chagas disease

and to persistence of the disease and possibly associated inflammation (Kahn *et al.*, 1996).

1.9.6. Diseases of unknown aetiology

1.9.6.1. Cardiovascular disease

There seems to be a delicate balance regarding when MBL may be harmful or beneficial in the cardiovascular system. A few examples are given below.

Chlamydia pneumoniae has been implicated as a significant contributory factor in atherosclerosis, and this has led to the suggestion that MBL deficiency could lead to an increased susceptibility to cardiovascular disease. Madsen *et al.* (1998) studied MBL genotypes of 76 Norwegian individuals with severe atherosclerosis and compared these to 100 Norwegian blood donor controls. The number of individuals homozygous for any variant allele was significantly increased in the patient group compared to the controls. In addition, there was a trend for those patients homozygous for variant alleles to be slightly younger at the time they first underwent a coronary by-pass operation compared to those patients who were heterozygous for a variant allele. Saevarsdottir *et al.* (2005) found in a cohort study in Iceland (including a cross-sectional group of 987 and a nested control sample of 1309 individuals) that the risk of developing myocardial infarction was higher in MBL deficient individuals.

In a study of Canadian Inuit, compared to geographically matched white controls, the Inuit were found to have an increased frequency of individuals that were wild-type for MBL alleles (Hegele *et al.*, 1999). The authors suggested that this might, in part, explain why the Inuit have a 40 % lower rate of cardiovascular disease than whites. However, the incidence of atherosclerosis in the groups studied was not determined. Recently, these results have been challenged in a prospective study of Caucasian males who developed atherosclerosis and controls matched for age and smoking history. This study found no difference in the median serum MBL levels between the two groups (Albert *et al.*, 2001). A more recent study demonstrated that in men high serum levels of MBL are associated with an increased risk of future coronary artery disease (Keller *et al.*, 2006).

Based on the idea of MBL as an initiator of inflammation, Biezeveld *et al.* (2003) studied the frequency of MBL genotypes in 90 Dutch patients with Kawasaki disease. They found a higher frequency of MBL mutations as compared to the genotypes in 88 controls ($p = 0.03$). In children younger than 1 year, those with mutations were at higher risk of developing coronary artery lesions (OR 16, $p = 0.026$). Kawasaki disease occurs more frequent in Oriental children (roughly 50/100,000, 10 times more frequent than in Caucasians (Royle *et al.*, 2005). Studying Kawasaki disease among Hong Kong Chinese patients Cheung *et al.* (2004) included 71 patients and 41 matched controls and determined MBL genotypes and MBL levels. They did not see a difference between the MBL genotypes of patients and controls. When analysing for brachioradial arterial stiffness, which is an important cardiovascular risk factor in the children, they found this to be associated with low MBL genotypes (multiple linear regression analysis, $p = 0.03$), and concluded that MBL genotype may modulate the disease. The recent finding of an association between a novel human coronavirus and Kawasaki disease (Esper *et al.*, 2005) may fit with the many reports of MBL having anti-viral activity.

Plaque material may be removed from inside the carotid artery (e.g., by endarterectomy) to avoid cerebral occlusion but restenosis often occurs after such a procedure (in typically 10% of the patients after the first year). In a prospective study of 123 patients Rugonalfi-Kiss *et al.* (2005) it was found that female patients with genotypes associated with lower MBL levels had a slower rate to early restenosis, suggesting that a high level of MBL may be part of the pathophysiology of this condition.

1.9.6.2. Recurrent miscarriage

MBL has also been associated with recurrent miscarriage. Low MBL concentrations were observed more frequently in couples with a history of recurrent miscarriage compared to obstetrically normal controls (Kilpatrick *et al.*, 1995). These findings were confirmed by Christiansen *et al.* (1999) who also observed that the degree of MBL deficiency correlated with the number of previous miscarriages. However another study failed to find any difference in MBL genotype between couples with idiopathic recurrent miscarriage and couples with no history

of miscarriage and at least one live birth (Baxter *et al.*, 2001). In patients with pre-eclampsia, which may share a common (but as yet undefined) genetic basis with spontaneous abortion, no significant difference was found between patients and blood donors (Kilpatrick, 1996).

1.9.6.3. Sudden infant death syndrome and premature birth

Kilpatrick *et al.* (1998) in an isolated study, sought evidence of a relationship between MBL concentration and Sudden Infant Death Syndrome (SIDS), and found that the mean concentration of the protein was increased in the SIDS children compared to live controls. This was attributed to an acute phase response in such patients and is consistent with the view that some cot deaths are preceded by infections.

Two recent studies demonstrated that fetal MBL2 genotype can be an additional genetic factor contributing to the risk of premature delivery (Bodamer *et al.*, 2006) and is associated with pre-term birth (Frakking *et al.*, 2006).

1.9.7. Autoimmune diseases

Evidence that the innate immune system could lead to autoimmunity, either by priming or by promoting aggressive immune responses, is mounting (Ezekowitz, 2003). A major current pathophysiological concept of autoimmunity is impaired apoptotic cell clearance. Boniotto *et al.* (2005) suggested that impaired removal of apoptotic cells due to MBL deficiency might predispose to the development of autoimmune symptoms. In *vitro* studies demonstrated the role of MBL in removal of apoptotic cells (Ogden *et al.*, 2001; Nauta *et al.*, 2004; Roos *et al.*, 2004). Mice lacking MBL exhibits less efficient removal of apoptotic cells in *vivo* (Stuart *et al.*, 2005).

1.9.7.1. Systemic lupus erythematosus

The development of Systemic Lupus Erythematosus (SLE) may be dependent upon both genetic and environmental factors. Deficiencies of the early classical pathway complement components C1q, C2 and C4 have long been known to be associated with SLE.

MBL associations with SLE have been sought by several groups in various populations In Chinese SLE patients the B allele frequency was found to be slightly increased compared to healthy Chinese controls (Lau *et al.*, 1996). A similar finding involving the C allele was observed in African-American patients compared to ethnically-matched controls (Sullivan *et al.*, 1996). An increased frequency of one or more mutant alleles was also described in association with SLE in UK Caucasians (Davies *et al.*, 1995), Danish (Garred *et al.*, 2001) and Spanish Caucasians (Davies *et al.*, 1997; Villarreal *et al.*, 2001). More recently, SLE has been shown to be associated with the low promoter polymorphism LX in Chinese patients compared to Chinese controls (Ip *et al.*, 1998).

In contrast to these findings no association was found between MBL mutations in Japanese SLE patients compared to normal controls (Horiuchi *et al.*, 2000).

Nevertheless, a recent meta-analysis has reviewed studies in this area and found that MBL variant alleles such as MBL exon 1 codon 54 B, promoter –550 L, and promoter –221 X are SLE risk factors (Lee *et al.*, 2005). Interestingly, in SLE patients, MBL deficiency increases the risk for respiratory tract infections (Garred *et al.*, 2001;Takahashi *et al.,* 2005) as well as the risk of developing arterial thromboses (Ohlenschlaeger *et al.*, 2004). A higher risk of cardiovascular disease and chronic renal failure was observed in SLE patients carrying MBL low genotypes (Font *et al.*, 2006). Furthermore, MBL-deficient SLE patients manifest increased levels of autoantibodies against molecules associated with apoptotic cells, such as cardiolipin (Seelen *et al.*, 2005). Autoantibodies to MBL are present in 8-24% of SLE patients although no significant correlation had been observed between their high titers and disease activity or specific clinical manifestations (Kravitz and Shoenfeld, 2006).

1.9.7.2. Rheumatoid arthritis

Studies of the association between MBL and rheumatoid arthritis have demonstrated that MBL is able to bind to rheumatoid factor (RF) complexes and assist their clearance by the reticuloendothelial system (Day *et al.*, 1980; Sato *et al.*, 1997).

In 1995, Malhotra and colleagues were able to show by nuclear magnetic resonance (NMR) and X-ray imaging that MBL is able to bind to the IgG-GO

glycoform (which lacks the terminal Fc region galactose and instead terminates in N-acetyl glucosamine and is increased in patients with rheumatoid arthritis) and subsequently activate and deposit more C4 compared to normal pooled IgG in *vitro*. Thus it was suggested that MBL participated directly in the disease process.

However, a subsequent study by Graudal *et al.* (1998) of Danish Caucasians found that significantly more rheumatoid arthritis patients had undetectable MBL compared to controls. In addition, patients with levels below the median were shown to be younger at the onset of their arthritis - a finding also supported in another study (Garred *et al.*, 2000). MBL also appeared to modulate disease since patients with MBL levels below the median had significantly higher scores in four out of seven parameters predictive of disease severity.

Further evidence for an association between MBL and rheumatoid arthritis was published by Ip *et al.* (2000) who showed that Southern Chinese patients with rheumatoid arthritis had significantly lower serum MBL levels than controls. Moreover, patients with erosive and serious extraarticular disease had lower MBL levels than those without such serious features.

Low MBL levels have also been shown to increase radiographic joint destruction (Graudal *et al.*, 2000), and contribute to a poor prognosis in those with early onset rheumatoid arthritis (Saevarsdottir *et al.*, 2001) although the latter study contradicted some of the findings mentioned above by not finding any difference in MBL mutations between patients and controls. Other studies have also reported that there is no association between MBL level / genotype and rheumatoid arthritis in the Japanese (Horiuchi *et al.*, 2000) and Caucasians (Stanworth *et al.*, 1998; Kilpatrick *et al.*, 1997). The latter study also showed that those patients with low or medium MBL genotypes did not differ from wild-type patients in the frequency of rheumatoid factor positive individuals or the occurrence of rheumatoid nodules. In the study by Garred *et al.* (2000) patients with late-onset and advanced disease who were wild-type for the MBL exon 1 allele were shown to have more inflammation compared to those with mutations in the exon 1 region suggesting that increased MBL levels may lead to inflammation due to an increase in complement deposition. The recent study detected the elevated levels of anti-MBL antibodies in the sera of RA patients, which can potentially have a diagnostic value (Gupta *et al.*, 2006).

1.9.7.3. Other autoimmune diseases

Sjögren's syndrome is an autoimmune disease characterised by a dry mouth and dry eyes due to attack by immune cells of the glands that produce tears and saliva. In a study of 104 Japanese patients with primary Sjögren's syndrome, there was a significant increase in individuals heterozygous or homozygous for the MBL B allele compared to healthy controls, suggesting a role for MBL in susceptibility to this disease (Wang *et al.*, 2001).

Celiac disease is a multifactorial disorder with a strong allergic reaction against gluten in the small intestine. The development is linked to the HLA haplotypes, DR2 and DR8. A study Boniotto *et al.* (2002) encompassing 117 patients and 130 controls indicated an association between celiac disease and the presence of variant MBL alleles. Later the same group (Boniotto *et* al., 2005) investigated 149 patients and 147 controls and found the frequency of homozygosity for variant MBL alleles to be higher in the patients ($p = 0.035$). The low MBL genotypes were strongly associated with more celiac disease symptoms ($p = 0.001$) as well with increased frequency of secondary autoimmune diseases ($p = 0.01$). By immunohistochemistry MBL was found to be present, together with apoptotic cells, in the basal lamina under the intestinal epithelium, where they had previously found mRNA for MBL (Boniotto *et al.*, 2003). This finding in Italy is supported by a study of Finnish celiac disease patients in which Iltanen *et al.* (2003) found an increased frequency of the B variant of MBL in a study of 88 celiac disease patients compared to 138 controls ($p = 0.004$)). It could be that increased susceptibility to intestinal infections and diarrhoea, associated with low MBL, may change the intestinal epithelia thus allowing for abnormal stimulation of anti-gliadin immune responses and triggering of the cascade leading to celiac disease.

The possibility of MBL involvement has also been considered in two other inflammatory bowel diseases (IBD), Ulcerative colitis (UC) and Crohn's disease (CD). Their pathogenesis is unknown, but genetic as well as environmental factors, e.g., microorganisms, are implicated. Rector *et al.* (2001) examined MBL genotypes of 431 IBD patients (142 UC and 287 CD and 2 with indeterminate colitis) and compared this with 112 affected and 141 non-affected first-degree relatives and 308 healthy controls. The number of individuals with low MBL variant

haplotypes was significantly lower in UC as compared to CD ($p = 0.01$) and to controls ($p = 0.02$), while no difference was found between CD and controls. It was suggested that MBL might be responsible for the more extensive complement-mediated mucosal damage in UC compared to CD and low MBL levels could thus protect somewhat against the development of the disease.

Seibold *et al.* (2004) conducting a smaller study, measured MBL levels in 74 CDs, 22 UCs and 32 healthy controls, and determined MBL genotypes in 58 CDs, 18 UCs and 47 controls (excluding patients with active disease from the study). The frequency of homozygous and compound heterozygous for variant exon 1 alleles differed significantly between patients suffering from CD or UC and the healthy controls ($p < 0.01$). If only CD patients and controls were considered, the significance increased further ($p = 0.005$). Antibodies against mannan from *Saccharomyces cerevisiae* were present in 47% patients with CD and in 0% of the controls. It was found that more CD patients with anti-mannan antibodies had low MBL levels ($p < 0.0001$) as was also true when looking at T-cell proliferation in response to mannan ($p < 0.0001$). It would appear that the immune reactivity against mannan in CD is regulated by the MBL concentration. Studies on MBL and IBD are few and it seems likely that profound (and potentially useful) information may be gained by extending these studies.

A recent study demonstrated that both MBL serum concentration and MBL complex activity were significantly higher in new-onset juvenile type I diabetic patients than in healthy controls (Bouwman *et al.*, 2005) suggesting that MBL might be involved in the pathogenesis of diabetes by assisting the autoimmune process of insulitis. A major source of mortality and morbidity in diabetes is caused by microvascular complications.

Several studies have demonstrated the association between the presence of high MBL producing genotypes and the increased risk of developing renal complications (Hansen *et al.*, 2004; Hovind *et al.*, 2005; Saraheimo *et al.*, 2005; Saevarsdottir *et al.*, 2005). Normoalbuminuric type 1 diabetics have been found to have higher MBL levels than non-diabetic controls, with a stepwise increase in circulating MBL levels with increasing levels of urinary albumin excretion (Hansen *et al.*, 2003). The risk of dying and developing the albuminuria were significantly higher among type 2 diabetic patients with higher MBL levels (Hansen *et al.*, 2006). In addition, MBL

deficiency may confer risk of obesity and insulin resistance (Fernandez-Real *et al.*, 2006).

1.9.8. Inappropriate activation of the lectin pathway

1.9.8.1. Nephrology

MBL has been implicated in the induction of glomerular injury in patients with IgA nephropathy via the activation of complement (Matsuda *et al.*, 1998; Endo *et al.*, 2001). The protein has also been found in the glomeruli of patients with glomerulonephritis, where it is believed to bind to agalactosyl oligosaccharides in a manner similar to that proposed for RA and resulting in complement deposition and inflammation (Endo *et al.*, 1998; Lhotta *et al.*, 1999).

More recently it has been suggested that this activation of complement may be caused by binding of MBL to IgA deposits, and this has subsequently been demonstrated *in vitro* (Roos *et al.*, 2001). Furthermore, in a study of renal biopsies from patients with IgA nephropathy MBL, MASP-1 and IgA2 were found to colocalise in the mesangium of all 19 patients studied suggesting that this may be the mechanism of lectin pathway activation in these patients (Hisano *et al.*, 2001).

One group has suggested that the MBL B allele may be important in IgA nephropathy, as this mutation was found at a significantly higher frequency in patients with glomerular IgA, IgG, IgM, C3 and C1q deposits than in those patients with only IgA and C3 deposits or in healthy controls (Gong *et al.*, 2001). However these findings were not confirmed in a slightly larger study which also investigated the B allele and found no difference in the allele frequency between patients and controls or different groups of patients when graded according to disease severity (Pirulli *et al.*, 2001). A more recent study demonstrated that 25% patients with IgA nephropathy show glomerular deposition of MBL, L-ficolin, MASP, and C4 (Roos *et al.*, 2006) and importantly, clinical and histological data clearly indicate that activation of the lectin pathway of complement is associated with more severe renal damage. Interestingly, MBL binds to polymeric IgA from patients with IgAN, which show significant increase in glycan composition and binding to mesangial cells (Roos *et al.*, 2006; Oortwijn *et al.*, 2006).

There is also evidence that MBL may play a role in kidney disease other than in IgA nephropathy. Indeed, MBL and MASP-1 have been detected in the glomeruli of patients with Henoch-Schönlein purpura nephritis, a vascular disease where glomerular injury is common (Endo *et al.*, 2000). In 16 patients with glomerulonephritis caused by the condition cryoglobulinemia – a complication of infections with hepatitis C virus – serum levels of MBL were found to be higher than in healthy controls and both MBL and MASP-1 could be visualised in the cryoprecipitate, again suggesting that MBL contributes directly to the inappropriate complement activation and the disease manifestations (Ohsawa *et al.*, 2001).

1.9.8.2. Ischemia/reperfusion injury

MBL had been implicated in increasing the amount of myocardial tissue injury incurred following reperfusion (Jordan *et al.*, 2001). Although inappropriate complement activation has long been known to contribute to this damage this study showed that pre-treatment of rats, before reperfusion, with an anti-MBL antibody significantly reduced markers of injury such as neutrophil infiltration and infarct size (Jordan *et al.*, 2001). Another study (Walsh *et al.*, 2005) has demonstrated that mice lacking MBL with fully active alternative and classical complement pathways are protected from cardiac reperfusion injury with resultant preservation of cardiac function. Significantly, mice that lack a major component of classical pathway initiation complex (C1q) but have an intact MBL complement pathway are not protected from injury. It is possible that IgM natural antibodies initially bind to ischemic antigens providing a binding site for MBL (Zhang *et al.*, 2006).

Tissue damage and impaired organ function resulting from ischemia/reperfusion injury still remain enormous impediments in solid organ transplantation. In support of MBL involvement in transplant-related I/R injury is the fact that MBL depositions were observed early after transplantation of ischemically injured kidneys (de Vries *et al.*, 2004).

1.9.8.3. Neurological diseases

It has been proposed that complement mediated attack could be involved in neuronal degeneration in Alzheimer's disease (AD), and as a result MBL has been implicated as a potential mediator of this inappropriate response. MBL has now

been found in the blood vessels of the brain in both AD sufferers and healthy controls (Lanzrein *et al.*, 1998). This study also measured serum and cerebrospinal fluid (CSF) concentrations of MBL and found no differences in the serum levels of the collectin but a significantly reduced concentration in the CSF of patients compared to controls. The authors postulated that this could be due to increased MBL consumption following inappropriate complement activation in AD patients. However no further studies have been performed to confirm or refute this. A recent study (Geleijns *et al.*, 2006) found that in Guillan-Barre syndrome, complement activation mediated by MBL contributes to the extent of the postinfectious immune-mediated peripheral nerve damage. The frequencies of the H allele, HY promoter and HYA haplotype were increased in 271 GBS patients compared with 212 healthy controls (Geleijns *et al.*, 2006).

1.9.9. MBL and non-infectious disease

1.9.9.1. Malignancy

MBL has been shown to recognise and bind to oligosaccharide ligands on human colorectal carcinoma cells and recombinant MBL was shown to inhibit growth of human colorectal carcinoma cells in mice (Ma *et al.*, 1999). However, it remains unclear whether there is any role for MBL in the immune response to tumours.

1.10. MBL deficiency and a possibility of therapy

Following the publication of many papers describing MBL disease associations and the interactions of MBL with various bacteria, it is logical to consider MBL replacement therapy in patients with severe infections associated with MBL deficiencies.

A small number of patients have so far been infused with MBL purified from pooled human plasma at concentrations sufficient to achieve normal serum levels. The MBL was safely administered with no side effects.

The first recipient, a 38-year old male, whose serum had a low MBL concentration and the common opsonic defect, had suffered from a range of diseases over many years including psoriasis and irritable bowel syndrome. Following infusions of MBL serum opsonic activity was restored and this was maintained until the

concentration fell to below approximately 300 ng ml^{-1} (approximately 50 hours post-infusion). The second patient, a 2-year old female with a history of recurrent infections from the age of 4 months, received infusions over three consecutive days and then three infusions at 10 days after the first infusion. She then remained healthy throughout the 3 year follow-up period apart from a minor sinusitis 4 months after the infusions (Valdimarsson *et al.*, 1998). Since the estimated half-life of MBL is 5 –7 days the long-term effects might simply reflect the recovery or maturation of other immune mechanisms made possible under the protective umbrella of MBL. A formal phase 1 trial involving 19 healthy volunteers has since been carried out (Valdimarsson *et al.*, 2004); the apparent safety of this plasma-derived product was confirmed. The biological half-life was rather variable in different individuals, but typically was only 2 to 3 days in contrast to 5 to 7 days for the first two subjects given purified MBL. This is surprisingly short, especially when MBL remains stable for up to 4 days in blood samples sequentially after death (Kilpatrick *et al.*, 1998). Plasma-derived MBL has been used sporadically on compassionate grounds in a few patients.

High expression of recombinant human MBL with biological activity can be obtained in Chinese hamster ovary cells (Ohtani *et al.*, 1999), and recombinant MBL produced in human embryonic kidney cells is allegedly equivalent to natural MBL (Vorup-Jensen *et al.*, 2001). Moreover, the similar product has been obtained in vivo after tail vein injection of mice with an MBL expression construct in the form of naked plasmid DNA (Vorup-Jensen *et al.*, 2001). A recombinant MBL would have several potential advantages over a plasma derived product: ease of large-scale production; freedom from the theoretical risks of viral or prion contamination; and possibly longer biological half-life. On the other hand, any recombinant MBL might have a different distribution of oligomers, and exhibit subtly different properties from the natural protein.

Phase I trials with rMBL have now been successfully concluded. There were no difference in incidence and type of adverse events reported between the groups of subject receiving rhMBL and the placebo group. Moreover, no anti-MBL antibodies were detected following rhMBL administration (Petersen *et al.*, 2006). While many of the MBL-deficiency-associated clinical conditions mentioned above would theoretically be candidates for reconstitution treatment, one must initially aim at

investigating small defined patient groups with relatively short follow-up periods of immunodeficiency, such as selected patients with chemotherapy-induced neutropenia or SIRS/sepsis patients (but obviously a better definition of the patients may be needed). The treatment of chronic disorders may possibly also be considered on the longer term.

1.11. Animal studies

The creation of MBL knockout mice has made possible experimental investigations of the effect of MBL deficiency. The mouse has two genes encoding different MBL molecules (MBL-A and -C) compared to one in humans. Both MBLs in mice are able to bind to carbohydrate surfaces and activate the complement system. A slight difference in carbohydrate specificity has been reported for the two mouse MBLs. Mice with only MBL-A knocked out were first produced (Lee *et al.*, 2002; Takahashi *et al.*, 2002), but only mice with both MBL-A and -C knocked out (MBL DKO) are suitable as an animal model of human MBL deficiency (Shi *et al.*, 2004).

In a sepsis model where *Staphylococcus aureus* was injected via the tail vein (Shi *et al.*, 2004), lack of MBL resulted in significantly increased mortality. Infusion of recombinant MBL reversed the phenotype. No difference was seen when the bacteria were injected intra-peritoneally. However, if the mice were treated with cyclophosphamide, simulating chemotherapy-induced neutropenia, before the intra peritoneal infection, the MBL DKO had more abscesses than the wild type. The MBL DKO mice were also more susceptible to challenge with herpes simplex virus type 2 (Gadjeva *et al.*, 2004).

In line with the suggested involvement of MBL in various autoimmune diseases the MBL DKO mice were examined for autoimmune symptoms when 18-month-old (Stuart *et al.*, 2005). No such signs were observed. On the other hand it was found that the ability to clear apoptotic cells was less efficient in the MBL knockouts. Another study evaluated the relative roles of C3 and MBL against *S.aureus* infection by generating MBLxC3 null mice in comparison with C3 single null mice (Takahashi *et al.*, 2005). It was found that an MBL deficiency had an additional effect on mice survival indicating that MBL-dependent mechanism in host resistance against *S.aureus* is C3 independent (Takahashi *et al.*, 2005).

It has been hypothesized that while MBL does not bind significantly to healthy tissue, changes due to abnormal conditions might reveal MBL ligands. Indeed, MBL is expressed by some tumour cell lines, and gene therapy with an MBL-vaccinia construct was found to be protective in nude mice transplanted with a human colorectal cancer cell line (Ma *et al.*, 1999). In vitro studies have indicated binding of MBL to cells exposed to hypoxia-reoxygenation (simulating ischemia/reperfusion) and subsequently it was shown that infusion of a blocking anti-MBL antibody would protect against myocardial destruction following ischemia/reperfusion in a rat model (Jordan *et al.,* 2001). Using MBL DKO mice Møller-Kristensen *et al.* (2005) found, in a model of kidney ischemia reperfusion (I/R) injury, that the MBL DKO were partially protected as evidenced by a better kidney function in these mice after ischemia/reperfusion. Increased deposition of the complement factor C3 was seen in wild type mice, and binding of MBL to sections of kidney could be inhibited with mannose. In agreement with this, de Vries *et al.* (2004) found MBL-A and -C deposited in the kidneys after ischemia/reperfusion in MBL wild type mice.

1.12. Aims of the investigations described in the thesis

The general aim of the present studies was to investigate the role of MBL and innate mechanisms in health and disease and the specific aims of the thesis were as follows:

1. To investigate MBL binding to selected microorganisms
2. To establish correlations between MBL haplotypes and plasma levels in general paediatric population
3. To evaluate the potential role of MBL in susceptibility to meningococcal disease, cystic fibrosis and SIRS
4. To study the effect of MBL on endothelial cells
5. To investigate the role of MBL in opsonophagocytosis

Chapter 2 General materials and methods

2.1. Reagents and Materials

The following reagents were used in the experimental work described. Unless otherwise stated all material was Analar grade.

Name	Company	Product code
Acetic acid	BDH	100016X
Accutase	PAA labs	L11-007
Agarose, electrophoresis grade	Gibco™ Invitrogen Corporation	15510-019
Ammonium chloride	BDH	100173D
Ammonium persulphate (APS), $(NH_4)_2S_2O_8$	Sigma	A9164
Ammonium sulphate	BDH	100336E
AmpliTaq Gold DNA polymerase	Perkin Elmer Applied Biosystems	N808-0241
Biotinamidocaproate N-hydroxysuccinamide ester	Sigma	B2643
Boric acid, H_3BO_3	Merck	20183.291
Bovine serum albumin, low endotoxin content	Sigma	A2934-100G
Bromphenol blue (3',3",5',5"tetra-bromophenolsulfonaphthalein), sodium salt, $C_{19}H_9Br_4O_5SNa$	Bio-Rad	161-0404
Calcium chloride (dehydrate), $CaCl_2x2H_2O$	Sigma	C3881
Cellfix (1% formaldehyde, 0.1% sodium azide)	Becton Dickinson	340181
Citric acid, C (OH) CO2H (CH_2CO_2H)	BDH	10081
Collagenase Type II	Gibco™ Invitrogen Corporation	17101-015

2'-Deoxynucleoside 5'-triphosphate (dNTP)	Promega	U1240
Dialysis tubing	Sigma	D9527
Dimethyl sulphoxide (DMSO)	BDH	103232
DMEM+L-Glu, 4500 mg L/D gluc	Gibco™ Invitrogen Corporation	41965-039
DNA ladder, 1 Kb	Gibco™ Invitrogen Corporation	15615-024
DNTP set 100mM	Invitrogen stores	10297018
Endothelial cell attachment factor	Sigma	E 9765
Ethanol, C_2H_5OH	Hayman	UN1170
Ethenediaminetetraacetic acid, di-sodium salt (EDTA), $C_{10}H_{14}N_2O_8Na_2x2H_2O$	Sigma	E5143
Ethidium bromide tablets, $C_{21}H_{20}N_3Br$	Sigma	E2515
FACSflow	Beckton Dickinson	342003
Fetal calf serum (endotoxin low)	Globepharm	F11962
Ficoll Paque Plus	Amersham Biosciences	17-1440-02
Film, scientific X-OMAT AR	Kodak	1651454
Fluoroscein isothiocyanate FITC	Sigma	F7250
Formaldehyde	Sigma	F8775
Galactose D-(+)	Sigma	G0750
Gentamicin	Roussel	Hospital stores
Gonococcal (GC) medium base	Oxoid	CM0367
Glycerol, $CH_2OHCHOHCH_2OH$	Sigma	G5516
Glycine, $C_2H_5NO_2$	Sigma	G7126
Hank's balanced salt solution with and without Ca^{2+} and Mg^{2+} salts	Gibco™ Invitrogen Corporation	24020-091 14025-050

Heparin sodium	CP Pharmaceuticals	PL4543/0208
Histopaque 1107	Sigma	1077-1
Hydrochloric acid, HCl	VWR	28507BF
Hydrogen peroxide (30% w/w), H_2O_2	Sigma	H1009
100bp ladder	Invitrogen stores	15628019
L-Glutamine 200mM (100x), liquid	Gibco™ Invitrogen Corporation	25030-024
Lipopolysaccharide, E.coli O111B: 4, gel purified	Sigma	L4391
Loading dye, 6x	Sigma	G7654
Magnesium chloride (hexahydrate) $MgCl_2.6H_2O$	Sigma	M8266-100G
Mannan-agarose	Sigma	M9917
Mannan, high grade	Sigma	M3640
Mannose D-(+)	Sigma	M6020
MCDB 131 medium	Gibco™ Invitrogen Corporation	10372-019
2-mercaptoethanol	Sigma	M6250
MilliQ water from Millipore Q plus purification system	Millipore	
Nitro-cellulose, ECL grade		
Paraformaldehyde $(CH_2O)_n$	Sigma	P6148
PBS tablets	Oxoid	BR0014g
PBS (Dulbecco solution)	Gibco™ Invitrogen Corporation	14190-094
Penicillin/Streptomycin	Gibco™ Invitrogen Corporation	15140-114
Protogel, containing acrylamide (30% w/v) and bisacrylamide (0.8% w/v)	National Diagnostics	EC-890
QIAMP DNA extraction kit (250)	Qiagen	51106

RPMI 1640 basal medium	Gibco™ Invitrogen Corporation	22511-026
RPMI 1640 medium with 25mM HEPES and 10mM L-glutamine	Gibco™ Invitrogen Corporation	52400-041
RPMI 1640 medium without phenol red	Gibco™ Invitrogen Corporation	32404-014
Sepharose 4B, CNBr activated	Amersham Biosciences	17-0981-01
Silver Stain SDS molecular weight standard mixtures	Sigma	M5505, M5630 and M6539
Silver stain protein detection kit	Bio-Rad	161-0449
Skimmed milk	Marvel	
Sodium azide, NaN_3	BDH	10369
Sodium bicarbonate, $NaHCO_3$	BDH	10247 4V
di-Sodium borate, $Na_2B_4O_7x10H_2O$	BDH	10267 4E
Sodium carbonate, Na_2CO_3	BDH	10240
Sodium chloride, NaCl	BDH	10241
SDS (sodium dodecyl sulphate [Lauryl sulphate])	Sigma	L4390
SDS-PAGE standards (biotinylated markers)	Bio-Rad	161-0311
SDS-PAGE standards (Rainbow markers)	Amersham Pharmacia	RPN 756
SnakeSkin™ Pleated Dialysis Tubing	Pierce	68035
Sodium hydrogen carbonate, $NaHCO_3$	BDH	102474V
Sodium di-hydrogen orthophosphate, NaH_2PO_4	BDH	301324Q
di-sodium hydrogen	Sigma	S0876-500G

orthophosphate, Na_2HPO_4		
Sodium hydroxide, NaOH	BDH	102524X
Streptavidin Horse Radish Peroxidase conjugate	Amersham Pharmacia	RPN 1231
Streptavidin phycoerythrin PE-Cy5	Pharmingen	554062
Sulphuric acid	BDH	10249
TEMED (N',N',N",N" tetramethylethylenediamine)	Sigma	67H0136
3,3',5,5'-Tetramethylbenzidine tablets	Sigma	T3405
Tissue culture plates, 24 wells	Corning (Costar)	3524
Tissue culture flasks, $25cm^2$, Primaria	Becton Dickinson	35 3813
Tris, (hydroxymethyl) aminomethane, $NH_2C(CH_2OH)_3$	Sigma	T6066
Tris ([hydroxymethyl]aminomethane) Borate EDTA buffer (10X)	Sigma	T4415
TRIZMA base	Sigma	T8524
Trisol RNA extraction solution	Gibco™ Invitrogen Corporation	15596-026
Trypan blue	Sigma	T6146
Trypsin-EDTA	Gibco™ Invitrogen Corporation	25300-054
Tween – 20 (polyoxyethylene (20) sorbitan monolaurate)	Sigma	P1379
Vitox	Oxoid	SR0090A
Vivaspin 15R	SLS	FIL8448

2.2. General buffers and solutions

General buffers	**Phosphate buffered saline (PBS), pH 7.3** NaCl 140.0 mM KCl 2.7 mM Na_2HPO_4 8.0 mM KH_2PO_4 1.5 mM Prepared by the addition of one PBS tablet to 100 ml MilliQ water
	PBS Tween Prepared as above with the addition of 0.5 % (v/v) Tween-20

2.3. Antibodies

Name	Company	Product code
Anti-human MBL, mouse monoclonal IgG clone 131-1	State Serum Institute, Copenhagen, Denmark	
anti-human CD31, mouse IgG1 monoclonal. Clone WM59	Serotec	MCA1738
anti-human CD62E, mouse IgG1 monoclonal. Clone 12.B6	Serotec	MCA1969
anti-human ICAM-1, mouse IgG1 monoclonal. Clone 84H10	Serotec	MCA773
Mouse IgG1 negative control	Serotec	MCA1209
Phycoerythrin	Dako	R0480

conjugated anti-mouse F $(ab')_2$ goat polyclonal.		
FITC conjugated anti-mouse F(ab')2 goat polyclonal	Dako	F0479
Monoclonal antibody to human C4d containing fragments, IgG1k	Quidel	A213
Mouse monoclonal antibody to an epitope in the Bb fragment of human Factor B; IgG1k	Quidel	A227

2.4. Purification of Mannose-binding lectin

2.4.1. Buffers

MBL purification Buffers	**Tris Buffered Saline (TBS), pH 8.0**	
	Tris	0.02 M
	Sodium chloride	0.1 M
	Sodium azide	0.02 % (w/v)
	Tris Buffered Saline with Calcium (TBS^+), pH 8.0	
	Tris	0.02 M
	Sodium chloride	0.5 M
	Calcium chloride	0.01 M
	Sodium azide	0.02 % (w/v)
	Elution buffer No. 1	
	TBS	
	EDTA	10 mM
	Elution buffer no.2	
	TBS	
	Mannose	100 mM

	Column washing buffer No. 1	
	Sodium borate pH adjusted to 10.0	0.1 M
	Column washing buffer No. 2	
	Glycine-HCl	0.1 M
	All solutions degassed and filtered using a 0.2 μm filter before use.	
Protein Electrophoresis Buffers	**10 x Transfer Buffer**	
	Tris	48.0 mM
	Glycine	39.0 mM
	3.75 ml 10 % (w/v) SDS added to 1l of 1 x transfer buffer before use	
	SDS-PAGE running buffer	
	Glycine	190.0 mM
	Tris	25.0 mM
	SDS	0.1 % w/v
	2 x Sample buffer	
	Tris	120.0 mM
	Glycerol	20.0 % (v/v)
	SDS	4.0 % (w/v)
ELISA Buffers	**ELISA coating buffer, pH 9.6**	
	$NaHCO_3$	35.0 mM
	Na_2CO_3	**15.0 mM**
	Citrate / phosphate buffer, pH 5.0	
	Citric acid ($C_6H_8O_7.H_2O$)	0.1 M
	Na_2HPO_4	0.2 M
	TMB ELISA substrate buffer	
	Citrate / phosphate buffer	**20.0 ml**
	TMB tablets	4
	H_2O_2	0.015 % (v/v)

2.4.2. Choice of purification method

The first purification of MBL was described by Kozutsumi *et al.* (1980). This preparation was obtained from rabbit serum applied to a column of mannan derived from the *Saccharromonas cerevisiae* cell wall and coupled to a solid support. In subsequent years many more purification techniques have been described for mouse (Hansen *et al.*, 2000), rabbit (Kozutsumi *et al.*, 1980) and human MBL (Nevens *et al.*, 1992; Tan *et al.*, 1996; Kilpatrick, 1997). Many of these techniques have used mannan or mannose affinity columns for purification, exploiting the carbohydrate binding properties of MBL. However, several issues arise regarding the purification of MBL by carbohydrate binding. Firstly, MBL is not the only molecule present in serum, which binds to mannan and mannose and therefore a single step purification by this technique often results in contamination with other molecules including immunoglobulins. Various recombinant human MBL proteins have been described but in several cases their physicochemical properties differed from the MBL purified from serum, raising the question of whether recombinant MBL should be used in functional studies (Lipscombe *et al.*, 1995). In addition, the recombinant protein is not associated with MASPs, resulting in the need for these proteases to be added from another source if the protein is to be used in functional studies.

After careful consideration of the above issues it was decided to purify human MBL using an affinity chromatography method. Three affinity chromatography columns were used to achieve purity. Essentially, the sample was twice passed through mannan-agarose columns to isolate the protein, and the product was then passed through an anti-immunoglobulin column as a safeguard against immunoglobulin contamination.

2.4.3. Source of human MBL

Cold ethanol fractionated pooled human plasma was used as a source of MBL and this was provided by the Blood Products Laboratory, Elstree, London, UK. This source was convenient as a large volume of starting material was required to obtain a high yield of MBL. Specifically, the B+1 fraction was provided as a paste and had the added advantage that it had been partially purified resulting in significant depletion of both albumin and IgG.

2.4.4. MBL purification

Five hundred grams of human plasma paste (B+1 fraction) were defrosted overnight at +4°C in 2 litres of TBS buffer. The defrosted mixture was then centrifuged at 12000 rpm for 20 min at +4°C in the Sorvall RC-5B centrifuge and the supernatant decanted. Ammonium sulphate was added to the supernatant to approximately 42 % saturation (130 g / 500 ml supernatant) and incubated at room temperature for 1 hour before being re-centrifuged as before. The supernatant was discarded and the precipitated protein pellets pooled together and dissolved in TBS^+ buffer to an approximate volume of 900 ml, which was then dialysed overnight at +4°C against TBS^+. Clotted paste was homogenised manually using a 50 ml syringe before the paste was re-centrifuged as before. Finally, the supernatant was decanted and stored at +4°C for affinity chromatography.

A water jacketed XR column (16/20) (Amersham Biosciences) packed with mannan-agarose was cooled to +4°C and equilibrated with 30 ml TBS^+ at 2 ml min^{-1} using a ACTA. The paste was applied to the column using the ACTA peristaltic pump at a rate of 0.5 ml min^{-1}, and the column then washed with TBS^+ at 2 ml min^{-1} until the absorbance at 280 nm returned to baseline levels (this and all further steps were performed using the P-500 pump). The column was eluted with TBS containing EDTA at a flow rate of 0.5 ml min^{-1}. Two millilitre fractions were collected until the elution peak was observed. The column was then washed with TBS^+ containing 100 mM mannose at 1 ml min^{-1}, followed by 0.1 M sodium borate, pH 10.0, and finally 30 ml of TBS^+ at 1 ml min^{-1} until the eluent absorbance at 280 nm returned to baseline.

Both an Amersham Biosciences HR mannan-agarose column (gel volume 10 ml, i.d.10mm) and an HR anti-IgG column (gel volume 5 ml, i. d. 5mm) were equilibrated with 30 ml of TBS^+ before sequential application of the eluent from the XR mannan- agarose column. Recalcified fractions were applied to the HR mannan-agarose column and washed through with TBS+ until the baseline returned to zero. The MBL from the first column was eluted with TBS+ + 100 mM mannose at 0.5 ml/min, collected into the superloop and then passed through the HR anti-IgG column. MBL was collected from the second column as a flow through fractions into 2 ml tubes. Both columns were washed twice with 5 ml of 0.1 M

Glycine-HCl, re-equilibrating with 30 ml TBS^+ after each wash before storing at +4°C.

The column flow-through fractions were pooled and concentrated into PBS + 0.02% NaN_3 using a Vivaspin 15R (Vivasciense). The material was then stored at +4°C until required.

2.4.5. Analysis of the purity of MBL obtained

The purity of each MBL preparation was determined by performing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing conditions, with final detection by silver staining or enhanced chemiluminescence (ECL) development of immunoblots (Lipscombe *et al.*, 1995). The concentration of MBL eluted from the anti-MBL column and further purified using the anti-IgG column was determined by an ELISA procedure.

2.4.5.1 SDS-Polyacrylamide gel electrophoresis of MBL under reducing conditions

For analysis under reducing conditions, 10 % acrylamide SDS-PAGE gels were prepared. Samples were diluted with an equal volume of 2 x sample buffer containing 4 % 2-mercaptoethanol (freshly prepared), and heated at 105°C for 3 min. They were then loaded onto the gel and electrophoresed at 120 V for 1 hr at room temperature alongside Rainbow Markers (size standards). Immediately after running, gels were fixed by incubating in 40 % methanol and 10 % acetic acid for at least 1 hr. Proteins were detected using the Bio-Rad silver stain detection kit according to the manufacturer's instructions. Development of the reaction was stopped by incubation of the gels with 5 % acetic acid.

2.4.5.2. SDS-Polyacrylamide gel electrophoresis of MBL under non-reducing conditions

For analysis under non-reducing conditions 3 – 10 % acrylamide continuous gradient SDS-PAGE gels were prepared using a Bio-Rad gradient former. Samples and biotinylated SDS-PAGE standards were diluted in an equal volume of 2x sample buffer (freshly prepared) and electrophoresed for 600 V alongside Rainbow Markers. Proteins to be detected by silver staining were fixed and processed as

described above. Proteins to be detected by enhanced chemiluminescence (ECL) were transferred from the gels to nitrocellulose membranes using a semi-dry transblotter (Trans-Blot, Bio-Rad, Hemel Hempstead, UK). Gels were carefully applied to the nitrocellulose membranes and sandwiched in between 6 pieces of filter paper, which, along with the membranes, had been pre-soaked in Transbuffer. Transfer was performed by electrophoresis at 20 V for 20 min. The membranes were then incubated in PBS containing 2 % skimmed milk for 1 hr at room temperature on a shaking platform to prevent binding of any free proteins to the membrane. Membranes were washed three times for ten minutes in PBS-Tween before being incubated in 1 $\mu g/ml^{-1}$ biotinylated anti-MBL in PBS-Tween for 3 hr at room temperature on a roller. Membranes were washed three times in PBS-Tween as before and then incubated in a 1 in 1000 dilution of Streptavidin-Horseradish peroxidase conjugate in PBS-Tween for 3 hr at room temperature on a roller. One final washing step was performed as before and the chemiluminescence reaction performed by applying the ECL detection reagents to the membranes for 1 min before draining and wrapping in Saran-Wrap. Proteins were visualised by exposure to X-ray film initially for 15 seconds, with options of longer discretionary exposures determined by the results of the initial exposure. Silver stained gels and ECL detected immunoblots were compared to assess the purity of the preparation.

2.4.6. Determination of the concentration of MBL in the purified preparations

A symmetrical sandwich ELISA was used as previously described to determine the concentration of purified MBL (Lipscombe *et al.*, 1996). At each incubation step a volume of 100 µl was used, all reagents were diluted in PBS containing 0.05% Tween-20 (PBS-T) unless otherwise stated, and each washing step consisted of three washes with PBS-T using a Denley Wellwash stacking ELISA plate washer. Immulon-2 plates were coated overnight at +4°C with a carbonate/bicarbonate coating buffer containing 1 µg/ml anti-MBL (clone 131-1), and then washed. Samples were generally diluted 1/20 or1/100 in PBS-T and calibrated against a doubling dilution standard curve based on a serum standard pool. Point standards of high, intermediate and low MBL sera were included in quadruplicate and test

samples were applied in duplicate. Serum dilutions were incubated at 37°C for one hour, washed and then bound MBL was detected using a biotinylated anti-MBL added at 1 μg/ml and incubated for one hour at 37°C. The plates were washed and a 1/1000 dilution of streptavidin-peroxidase solution was added and incubated for one hour at 37°C. After washing, the ELISA was developed using 100 μl TMB substrate solution. Adding 100 μl 4N H_2SO_4 stopped the reaction and the A_{450} of each well was read using a Dynatech MRX plate reader (Acterna, Aldermaston, UK) with the standards plotted as a sigmoid curve (Revelation software).

2.5. MBL protein levels

MBL levels in serum and in plasma were determined by a symmetrical sandwich Oligomer ELISA kit from the AntibodyShop, Copenhagen, Denmark according to the manufacturer's instructions.

2.6. Antibody conjugation procedures

2.6.1. Buffers

FITC conjugation of antibody

'A' mix

Na_2CO_3 (anhydrous)	5.8 ml	5.3% (w/v)
$NaHCO_3$	10.0 ml	4.2% (w/v)

Conjugation buffer

'A' mix	1 part
0.1 M NaCl	9 parts

pH of final mixture 9.5

2.6.2. Antibody biotinylation

Antibodies to be conjugated were equilibrated with 0.1 M sodium hydrogen carbonate prior to biotinylation. The reagent for biotinylation, biotinaminocaproate N-hydroxysuccinamide ester, was dissolved in DMSO to produce a concentration of 1 mg ml^{-1}, and the antibody was incubated with this solution (75 μl ester solution per milligram of antibody) for approximately 5 hours at room temperature. Following the incubation period the product was washed three times with PBS. The

final biotinylated antibody was aliquoted and stored at -70°C. Aliquots were defrosted prior to use and stored at +4°C for up to 1 month, to prevent freeze-thaw disruption, before being discarded.

2.6.3. FITC conjugation of antibodies

Antibodies to be conjugated were spun at low speed for 5 minutes and approximately 90 % of the supernatant removed by aspiration. The antibody was then made back up to the original volume using the FITC conjugation buffer (see section 2.2.) and this was repeated three times. FITC was dissolved to a concentration of 1 mg ml^{-1} in FITC conjugation buffer and 0.3 ml of this added per 1 ml of antibody. The mixture was incubated at room temperature for 3.5 hours to allow conjugation to take place. Unbound FITC was removed by washing the conjugated antibody with PBS until the product became clear (at least three washes).

The concentration of the antibody and the coupling efficiency were calculated by measuring the absorbance at 280 nm and 495 nm according to the equations below.

$$\text{Antibody concentration} = \frac{A_{280} - (A_{495} \times 0.35)}{1.35} \text{ mg ml}^{-1}$$

$$\text{Coupling efficiency} = \frac{A_{280}}{A_{495}}$$

A coupling efficiency ratio of 0.5 – 1.0 indicated good coupling whilst a ratio of less than 0.5 indicated a poor conjugation.

2.7. Genotyping

Genotyping was performed separately for the exon 1 mutations and the promoter polymorphisms using procedures similar to those previously described (Jack *et al.*, 1997; Turner *et al.*, 2000). In both cases a synthetic DNA molecule (Universal Heteroduplex Generator – UHG) was constructed spanning the region of interest and containing insertions, deletions and / or substitutions so as to permit detection

of all possible known mutations (kindly donated by Dr. N. Wood, University of Bristol).

2.7.1. Buffers

DNA electrophoresis buffers	**10x Tris-borate EDTA (10xTBE)**		
	Tris	0.9 M	108.00 g/l
	Boric acid	0.9 M	55.00 g/l
	Na_2EDTA	20.0 mM	7.44 g/l
	Working solution was diluted as appropriate		

2.7.2. Preparation of DNA samples for genotyping

Samples for genotyping were provided as whole blood in EDTA. Blood samples were stored at -20°C. DNA was extracted from 200 µl of whole blood using the Qiagen Blood Amp kit according to the manufacturer's instructions and the final product resuspended in 200 µl of sterile water before being stored at -20°C.

2.7.3. PCR amplification of DNA for MBL genotyping

Separate PCR reactions were performed on the genomic DNA samples, one to amplify the region of the gene containing the Exon 1 mutations, and one spanning the region of the promoter polymorphisms. Both of these PCRs were also performed for the synthetic UHG.

Genomic DNA was PCR-amplified for exon 1 in a 20 µl reaction using 5 µl of aqueous DNA (approximately 10 ng μl^{-1}) 0.6 µl each of 50 µM RMBL (5'-CCAACACGTACCTGGTTCC-3') and LMBL (5'-CTGTGACCTGTGAGGATGC-3') primers (Sigma-Genosys, Cambridge, UK), 2 µl of 10x PCR buffer (provided with AmpliTaq Gold enzyme), 2 µl of dNTP mix (2 mM each of dATP, dCTP, dGTP and dTTP), 1.2 µl of 25 mM $MgCl_2$ (provided with AmpliTaq Gold enzyme), 0.2 µl of AmpliTaq Gold DNA polymerase (5u μl^{-1}), and sterile water to make the total volume up to 20 µl. The exon 1 UHG amplification was carried out in volumes of 2000 µl with all reagents in the same proportions to the 20 µl genomic reactions except for the UHG DNA when 27 µl volumes were used. PCR reactions for the promoter polymorphisms were carried out in a similar fashion to the exon 1

procedure, the only exception being substitution of the primers for MBLproR (5'-CAGGGCCAACGTAGTAAG-3') and MBLproL (5'-CTAAGGAGGGGTTCATCTG-3') (Sigma-Genosys, Suffolk, UK) and primers for the X/Y promoter MBLproBL (5'-CTAAGGAGGGGTTCATCT-3') and MBLproRF (5'-AGGCATAAGCCAGCTGGCAAT-3').

PCR reactions were carried out at 94°C for 15 min, followed by 35 cycles (for exon1 and X/Y promoter) or 42 cycles (for full promoter PCR) of 94°C for 45 sec, 56°C for 45 sec and 72°C for 45 sec. These cycles were followed by a final extension step of 72°C for 10 min.

The presence of PCR product was confirmed by running 3 µl of product with 1 µl of bromophenol blue loading dye on a 2% agarose gel containing 0.5 µg/ml ethidium bromide in 1 x TBE running buffer for 1 hour. Gels were visualised under UV light.

2.7.4. Heteroduplexing and genotyping

Following confirmation of amplification of genomic DNA, 10 µl of UHG PCR product and 5 µl of loading dye were added to each sample. Samples were heteroduplexed by heating at 95°C for 10 min and then allowing samples to cool to room temperature for 20 min.

Fifteen microlitres of heteroduplex product were loaded onto polyacrylamide gels (20% for exon 1 and X/Y promoter analyses or 10% for the full promoter analyses) and run for 15 hr at 150 V in 1 x TBE buffer at 16°C (exon 1 and X/Y promoter) or 4°C (full promoter). Gels were stained in 0.5 µg/ml ethidium bromide in 1 x TBE buffer for 10 min before being visualised under UV light.

Figure 2-1 represents the typical pattern for exon 1 polymorphisms. Figures 2-2 and 2-3 represent typical patterns for promoter polymorphisms.

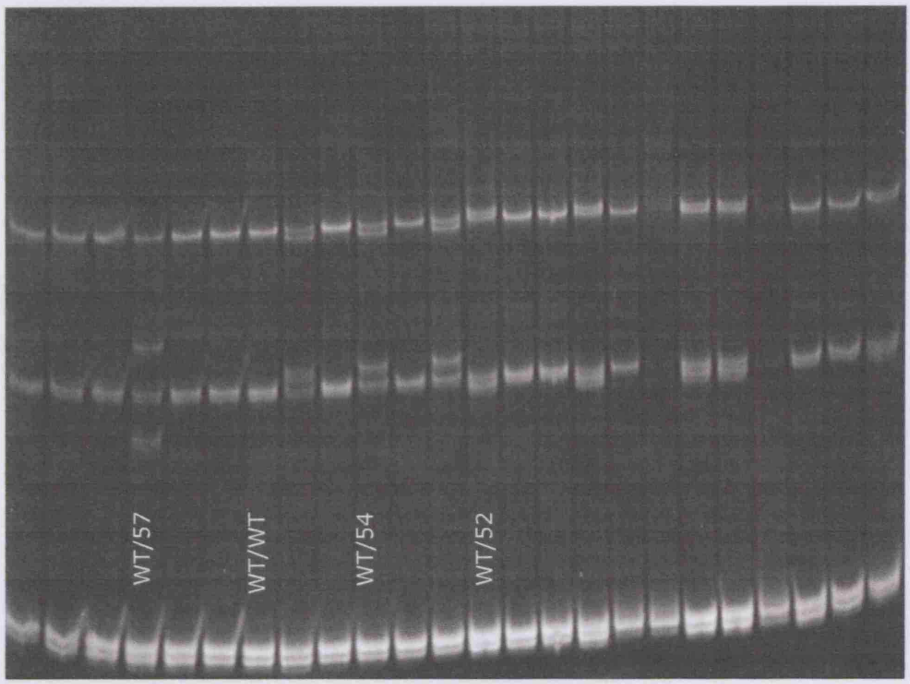


Figure 2-1. Photograph of polyacrylamide gel analysis of heteroduplex genotyping for exon 1 mutations in the MBL2 gene.

The typical band patterns representing different MBL exon 1 mutations can be easily identified. The labelled lines illustrate binding patterns characteristic of Variant C heterozygocity (WT/57), A variant wild type (WT/WT), Variant B heterozygocity (WT/54) and Variant D heterozygocity (WT/52). Separate polyacrylamide gels were run in order to identify promoter polymorphisms. Better band resolution was achieved when these gels were run at 16°C and some typical band patterns are shown in Figure 2-3.

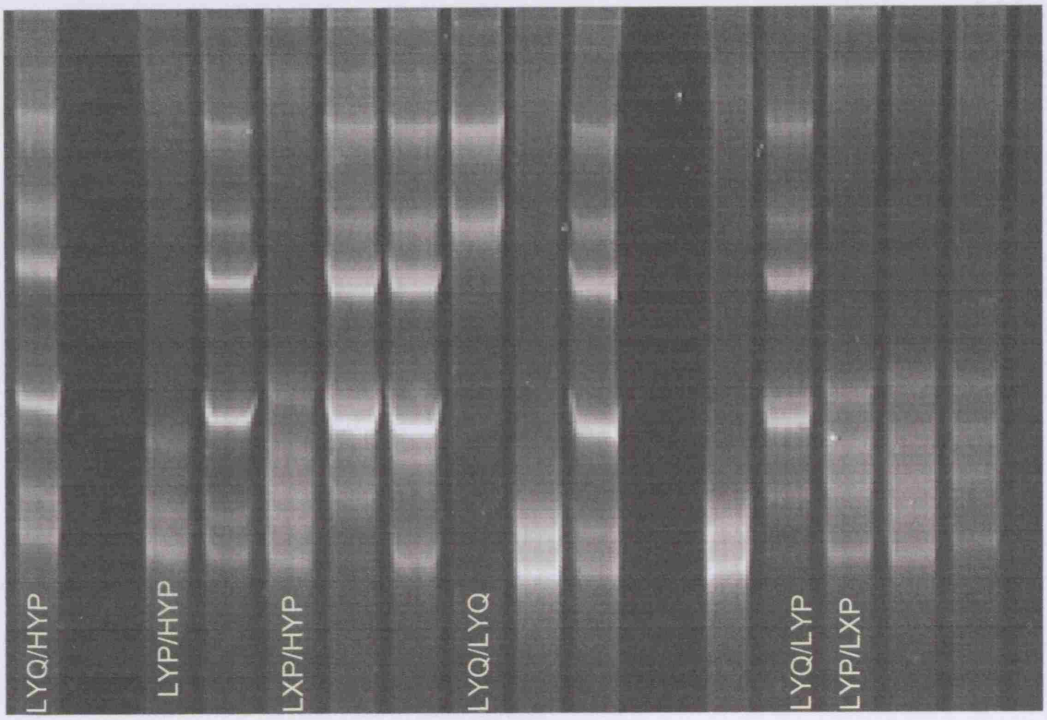


Figure 2-2. Photograph of polyacrylamide gel analysis of heteroduplex genotyping for all MBL2 gene promoter polymorphisms.

In some studies the X/Y promoter variation was determined instead of the full promoter profile. Some typical band patterns for these analyses are shown in Figure 2-3.

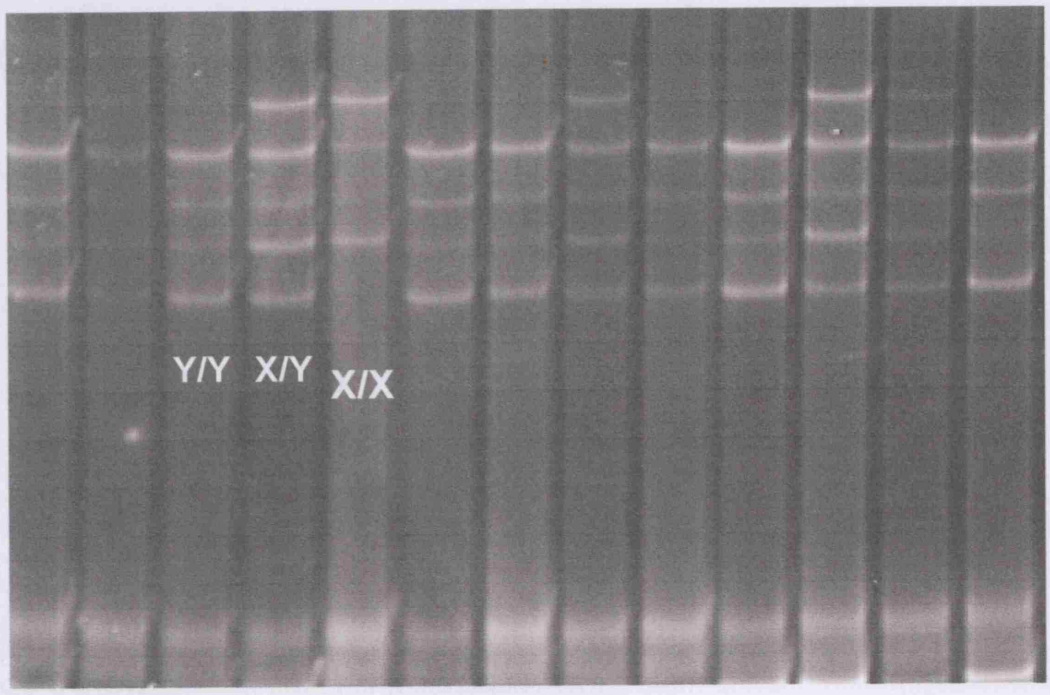


Figure 2-3. Photograph of polyacrylamide gel analysis of heteroduplex genotyping for the X/Y promoter variation in the human MBL2 gene.

Patterns characteristic of Y/Y homozygous, X/X homozygous and X/Y heterozygous individuals are illustrated.

Chapter 3 MBL binding to selected micro-organisms

3.1. Introduction

The acquisition of detailed knowledge of the fine structure of MBL has been paralleled by a series of publications reporting significant binding of this collectin to particular microorganisms. Such reports have presented evidence of MBL binding to individual pathogens, including human immunodeficiency virus type I (Ezekowitz *et al.*, 1989; Haurum *et al.*, 1993) and influenza A virus (Hartshorn *et al.*, 1993), and to the yeasts *C. albicans* and *C. neoformans* (Schelenz *et al.*, 1995; Tabona *et al.*, 1995). There have also been studies of MBL binding to various bacteria such as *Salmonella enterica* serovar Montevideo (Kuhlman *et al.*, 1989), *E.coli* (Kawasaki *et al.*, 1989), *Mycobacterium avium* (Polotsky *et al.*, 1997), and *Neisseria meningitidis* (Jack *et al.*, 1998; Jack *et al.*, 2001; Estabrook *et al.*, 2004). A previous study at Great Ormond Street Hospital, London has shown that MBL is able to bind to a wide range of clinically relevant bacteria and yeasts that commonly cause infections in children (Neth *et al.*, 2000).

Multiple determinants of MBL binding have been identified. Bacterial encapsulation has been shown to significantly inhibit the binding of MBL to a range of organisms associated with meningitis, such as *Neisseria meningitidis*, *Haemophilus influenzae*, different strains of *Streptococcus* and *Listeria monocytogenes* (van Emmerik *et al.*, 1994). The further studies with *N. meningitidis* have indicated that LPS structure, rather than and independently of encapsulation, may be the major determinant of MBL binding to these bacteria (Jack *et al.*, 1998). This is supported by other studies, which have also suggested that LPS structure and composition are important in determining the binding of MBL to bacteria (Ihara *et al.*, 1982; Ihara *et al.*, 1991; Kawakami *et al.*, 1982; Kuhlman *et al.*, 1989).

In our previous investigations, we have studied the influence of LPS structure on the binding of MBL to *Salmonella* serovar *Typhimurium* and *Neisseria gonorrhoeae*. The expression of a full-length (*Salmonella* serovar *Typhimurium*) or fully sialylated (*Neisseria gonorrhoeae*) LPS was required to prevent MBL binding (Devyatyarova-Johnson *et al.*, 2000). The expression of some truncated LPS

structures permitted the binding of MBL, but the identity of the terminal sugar was not always a reliable predictor of MBL binding.

Further studies were performed to explore determinants of MBL binding to bacteria with an emphasis on the role of LPS. For this purpose the following organisms were selected:

(a) *Neisseria meningitidis*

(b) *Helicobacter pylori*

(c) *Mycoplasma hominis*

(d) *Proteus mirabilis*

3.2. Materials and methods

3.2.1. Bacterial strains

3.2.1.1. Strains of *Neisseria meningitidis*

A series of isogenic mutants of the organism *Neisseria meningitidis* were a gift from Dr. L. Steeghs from the Department of Immunology, University Medical Centre Utrecht, The Netherlands. The parent organism *Neisseria meningitidis* serogroup (H44/76) was a piliated and encapsulated Norwegian isolate from a case of fatal septicaemia (Holten, 1979) and when grown as described in Section 3.2.2.1 it possesses a full-length lipo-oligosaccharide (LOS) (Figure 3-1).

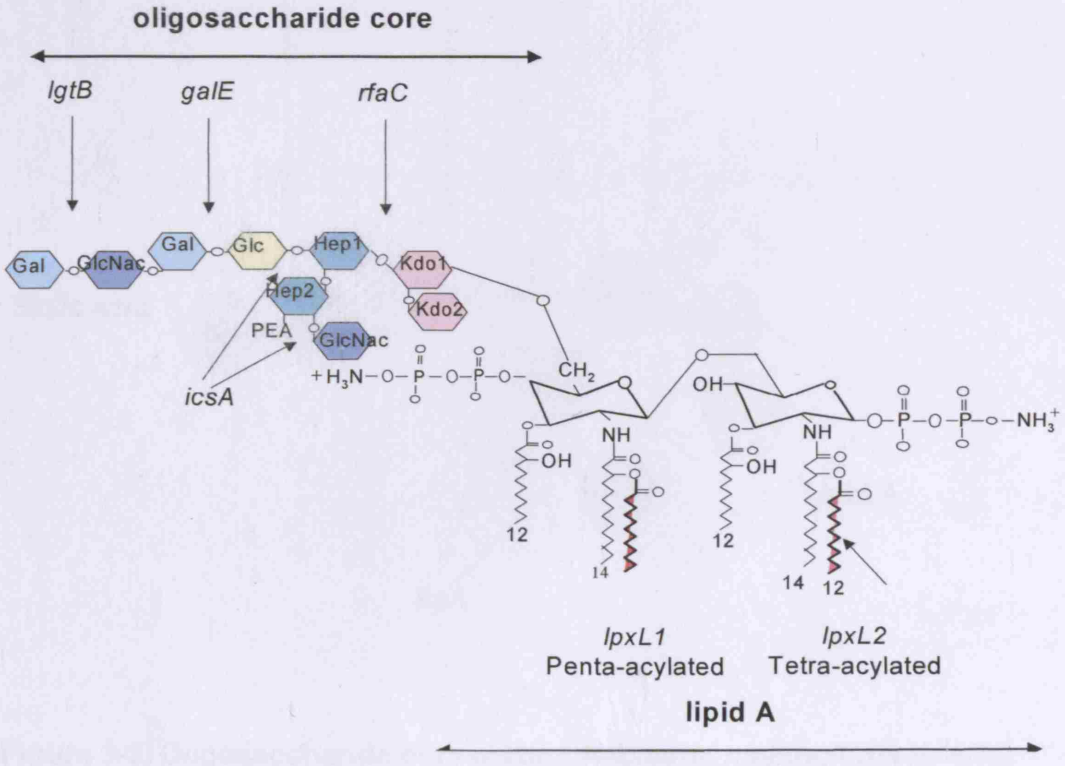


Figure 3-1. Schematic structure of *Neisseria meningitidis* H44/76 strain LPS immunotype 3 used in this study.

The zigzag structures denote the lipid anchor in the bacterial cell membrane. Open hexagons represent single saccharides with the oxygen atom identified by the open circle. Gal-galactose, GlcNAc – N-acetyl-glucosamine, Glc-glucose, Hep-heptose, KDO-keto-deoxyoctonate.

The oligosaccharide core (Jennings *et al.*, 1993; Jennings *et al.*, 1995; Pavliak *et al.*, 1993; Stojiljkovic *et al.*, 1997; van der Ley *et al.*, 1997) and lipid A (Steeghs *et al.*, 1998; Steeghs *et al.*, 2002; van der Ley *et al.*, 2001) mutant bacterial strains have been described previously. Structures of the oligosaccharide core mutants used in the studies outlined in this chapter are shown in Figure 3-2 and the lipid A mutants are illustrated in Figure 3-3.

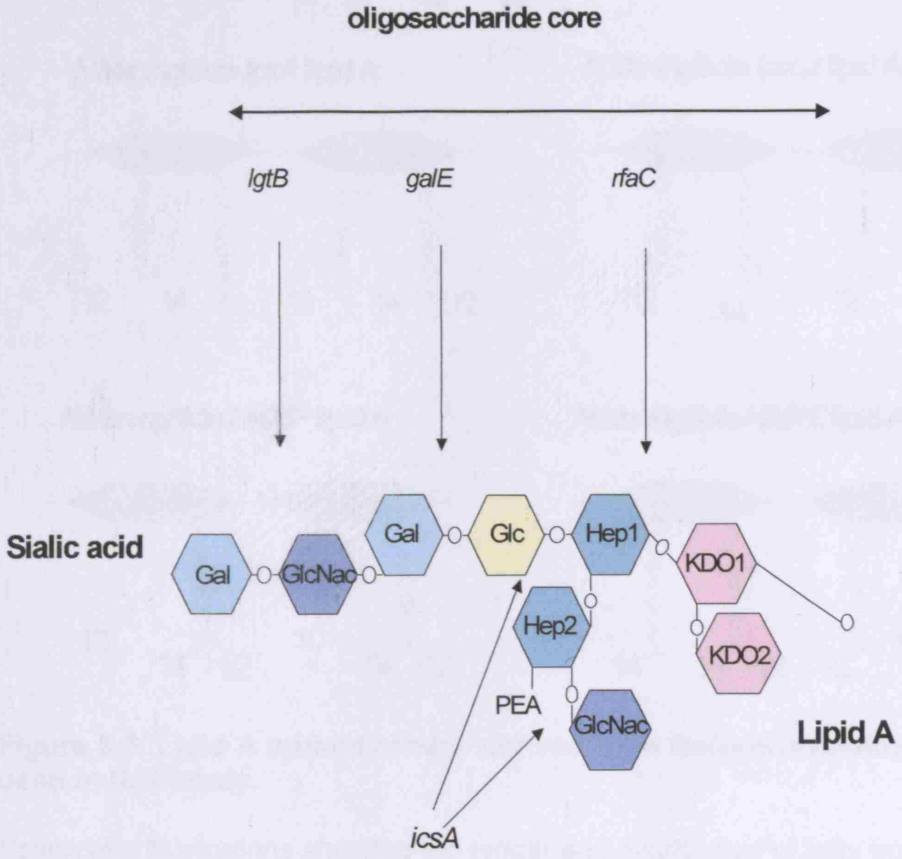


Figure 3-2. Oligosaccharide core mutant *Neisseria meningitidis* H44/76 strains used in this study.

The wild type expresses the full length sugar chain. The *lgtB*, *galE*, *icsA* and *rfaC* strains were obtained by genetic engineering to inactivate the genes required for oligosaccharide chain biosynthesis. The *siaD* mutant does not possess a capsule.

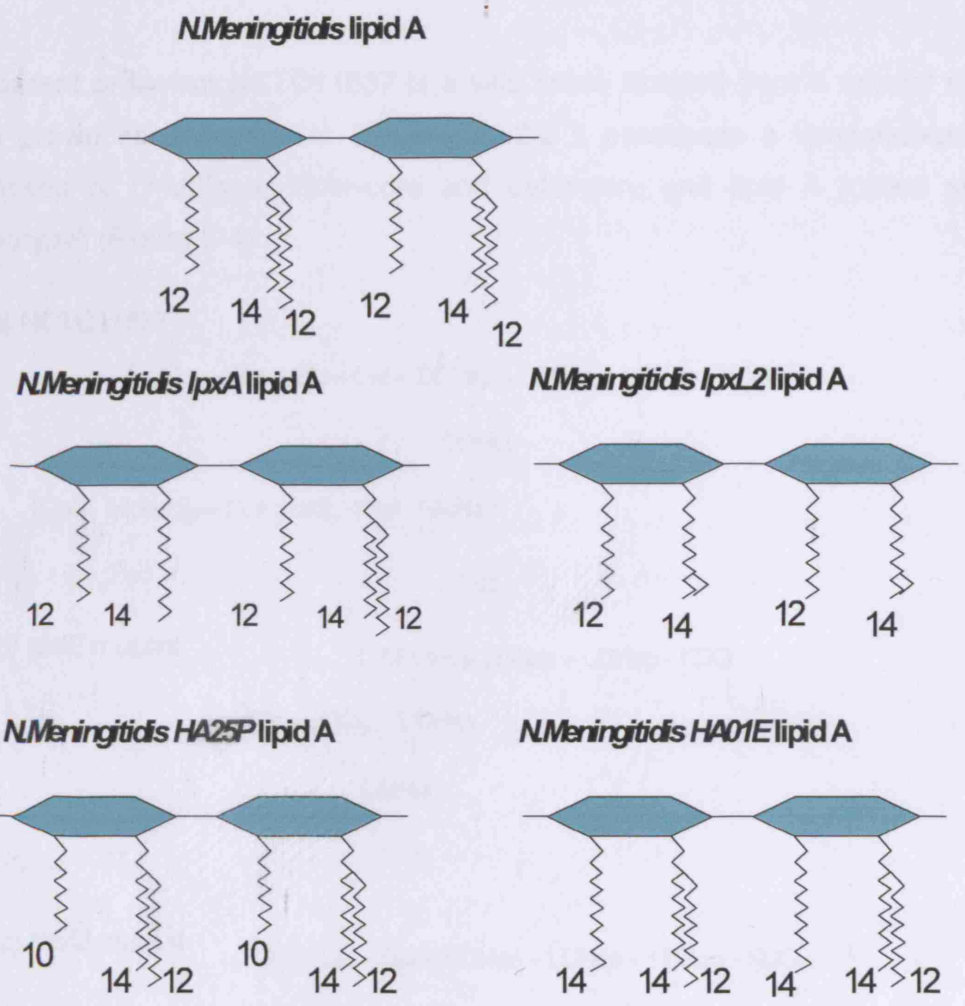


Figure 3-3. Lipid A mutant strains derived from *Neisseria meningitidis* H44/76 used in this study.

Schematic illustrations showing the length and distribution of fatty acyl chains in the *N.meningitidis* strains used in this study. An additional mutant, *lpxL1 galE* was also used. This strain has the same lipid A structure as the *lpxL1* mutant but expressed a truncated *galE* oligosaccharide chain, as did the *lpxL2* (see Fig. 3-1). The wt *N.meningitidis* expresses a symmetrical, hexa acylated lipid A and is shown on the top. Numbers indicate the quantity of carbon atoms in each fatty acid chain.

3.2.1.2. Strains of *Helicobacter pylori*

Two isogenic mutants of the organism *Helicobacter pylori* NCTC11637 were a gift from the Dr. Ben J. Appelmelk, Department of Medical Microbiology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands. The parent organism

and two mutants derived from it have been described previously (Edwards *et al.*, 2000).

The parent organism NCTC11637 is a wild strain isolated from a natural source; when grown as described in Section 3.2.2.2 it possesses a lipopolysaccharide composed of O-antigen, inner-core and outer-core and lipid A (called smooth chemotype) (Figure 3-4).

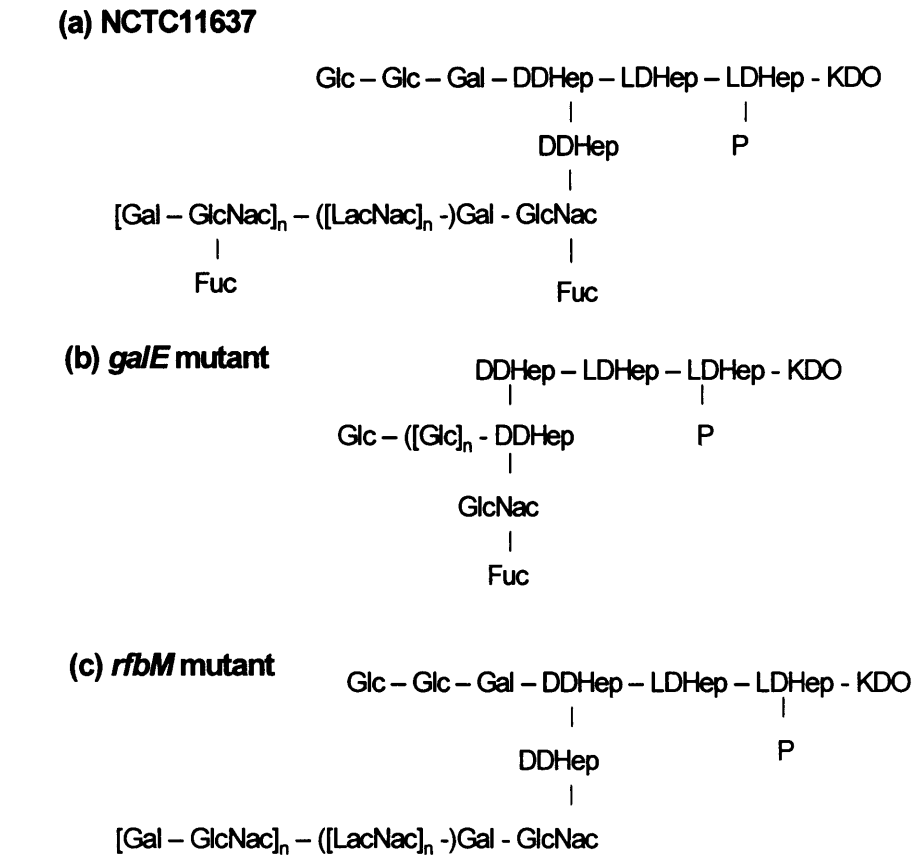


Figure 3-4. The proposed chemical structure of the dominant LPS expressed by (a) *H.pylori* NCTC11637; (b) galE mutant and (c) rfbM mutant.

Glucose -Glc, Heptose - Hep, Galactose - Gal, N-acetylglucosamine - GlcNac, Fucose -Fuc, P – phosphate and 2-keto-3-deoxyoctulosonic acid -KDO. Reproduced from N.J.Edwards *et al.*, 2000.

Mutants expressing truncated LPS structures were generated through insertional mutagenesis of *rfbM* and *galE*; genes encode GDP mannose pyrophosphorylase and galactose epimerase respectively. Compositional and structural analysis revealed that the *galE* mutant expressed a rough LPS that lacked an O antigen

side-chain. In contrast, an O antigen side-chain was still synthesized by the *rfbM* mutant, but it lacked fucose and no longer reacted with anti-Le^x monoclonal antibodies (Edwards *et al.*, 2000).

3.2.1.3. Strains of *Mycoplasma* organisms

Mycoplasma A39 (NCTC 11740) and *M. pneumoniae* (strain 5167) were obtained from Mycoplasma Experience (ME) Ltd, Reigate, UK. *M.hominis* (NCTC 10111) and *M.orale* were clinical isolates and were provided by Dr. D.Webster (Royal Free Hospital).

3.2.1.4. Strains of *Proteus mirabilis*

The strains of *Proteus mirabilis* were a gift from Dr. B.Senior, Department of Medical Microbiology, University of Dundee Medical School, Dundee, Scotland.

3.2.2. Growth and preparation of bacteria

3.2.2.1. Growth and preparation of *Neisseria meningitidis*

The strains were removed from storage at –70°C and cultured overnight at 37°C in 6% CO_2 on 3.6 % GC agar (36 g/l) plates supplemented with Vitox according to the manufacturer's instructions. Organisms were subcultured at least once. Immediately prior to each experiment organisms were suspended at a concentration of 10^8 organisms/ml in $HBSS^{++}$ (Appendix 1).

3.2.2.2. Growth and preparation of *Helicobacter pylori*

The strains were removed from storage at –70°C and cultured for 48 hours at 37°C on blood agar medium composed of Columbia agar base supplemented with 10% (vol/vol) defibrinated horse blood and *H.pylori* DENT supplement (Oxoid) according to manufacturer's instructions. Immediately prior to each experiment organisms were suspended at 10^8 organisms/ml in $HBSS^{++}$.

3.2.2.3. Growth and preparation of *Mycoplasma* organisms

All *Mycoplasma* organisms were cultured at 36°C in 50 ml volumes of Mycoplasma liquid medium (ML5A) supplied by Mycoplasma Experience or SP4 broth (made in house). Cultures were harvested when the density of *Mycoplasma* organisms was

sufficient, as indicated by a colour change (Poveda and R. Nicholas. 1998). *Mycoplasma* serovar hominis strain was cultured in Mycoplasma liquid medium (Mycoplasma Experience) at 36°C for 48-72 hours. Growth was again detected by colour (pH) change.

3.2.2.4. Growth and preparation of *Proteus mirabilis*

The strains were removed from +4°C and cultured overnight at 37°C on blood agar medium composed of Columbia agar base supplemented with 10% (vol/vol) defibrinated horse blood. Immediately prior to each experiment organisms were suspended at 10^8 organisms/ml in $HBSS^{++}$.

3.2.3. Assay for MBL binding to micro-organisms

3.2.3.1. Procedure for evaluating MBL binding

A 50 µl aliquot of organism suspension was spun at 9470 x g for 2.5 minutes. The supernatant was removed and the pellet resuspended in $HBSS^{++}$ containing MBL of different concentrations. Suspensions were incubated at 37°C for 30 minutes, and again centrifuged at 9470 x g for 2.5 minutes. The supernatant was removed and the pellet washed with 200 µl $HBSS^{++}$ before resuspension in 25 µl $HBSS^{++}$ containing 10 µg/ml FITC conjugated anti-MBL. The mixture of anti-MBL/organism was incubated at 37°C for 30 min, before spinning at 9470 x g for 2.5 min. The supernatant was removed and the pellet was washed with 200 µl of $HBSS^{++}$ and resuspended in 200 µl of PBS. The samples were fixed by the addition of 200 µl of Cellfix (Appendix I). The experiments were performed on live organisms.

3.2.3.2. Assay for MBL binding to *Mycoplasma* organisms

The binding of MBL to bacteria was determined by a modification of a flow cytometric procedure described in section 3.2.3.1. Briefly, a 2 ml aliquot of organism suspension was centrifuged at 12,000 g for 2 minutes in a microcentrifuge, and the pellet was resuspended in HBSS w/o phenol red containing MBL at 5 µg/ml concentration. Following further centrifugation at 12,000 g for 2 minutes, the cell pellet was washed with 200 µl of $HBSS^{++}$ before resuspension in HBSS containing FITC-anti-MBL. After incubation at 37°C for 30

minutes, the *Mycoplasma* preparations were pelleted (12,000 g, 2 min) and washed.

3.2.4. Specificity of MBL binding

The calcium dependence and sugar specificity of binding was investigated by incubating the organisms with the MBL preparation for 30 minutes at 37°C in the presence of between 10 to 100 mM of either EDTA, or galactose, or N-acetyl glucosamine, or mannose. MBL binding was then detected as in Section 3.2.3.1.

3.2.5. Flow cytometry

Live organisms were diluted in 200 μl of PBS containing 200 μl of Cell-Fix (in the proportion of 1:10). Organisms were selected on the basis of size and granularity. An aliquot of 10 μl of propidium iodide was added to each sample of *Mycoplasma* organisms before analysis. Organisms were identified by forward and side scatter to select particles of between 0.4 and 0.6 μm. Further gating to include only propidium iodide positive events was used to exclude non-bacterial particles.

Flow cytometry was performed on a FACSCalibur at low rate using CellQuest software (Becton Dickinson, Cowley, UK). Typical instrument settings are listed in Appendix I.

3.2.6. Statistical analyses

Kruskal-Wallis H-tests were used to determine the significance of differences in MBL binding. Probability was based on an analysed null hypothesis of differences in MBL binding between control and experimental populations. 'Significant' differences between control and sample populations were assessed at the $P<0.05$ level. A non-parametric test was used due to the small sample size ($N<30$).

3.3. Results

3.3.1. MBL binding to different organisms

3.3.1.1. Binding MBL to *Neisseria meningitides* H44/76

In the present study, flow cytometry was used to investigate the interaction of MBL with a strain of the organism, *Neisseria meningitidis H44/76,* and eight isogenic mutants. Using flow cytometry it was possible to detect a distinct population of events based on forward and side scatter. Events were collected on the basis of size and granularity, as shown in Figure 3-5.

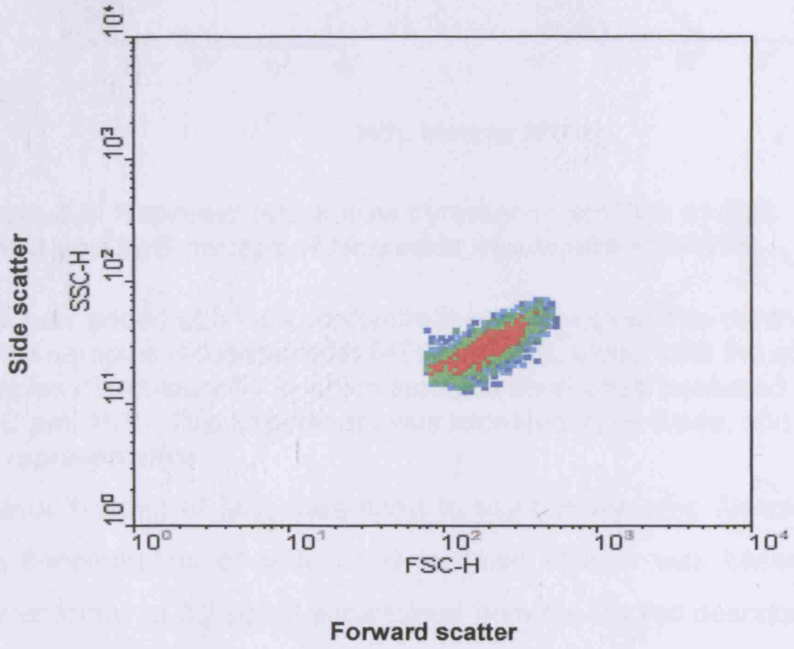


Figure 3-5. Characteristics of *Neisseria meningitidis H44/76* organisms on flow cytometry.

The *Neisseria* organisms were identifiable as a single population on forward and side scatter. In all experiments an event had to fulfil the conditions of being the correct size and granularity.

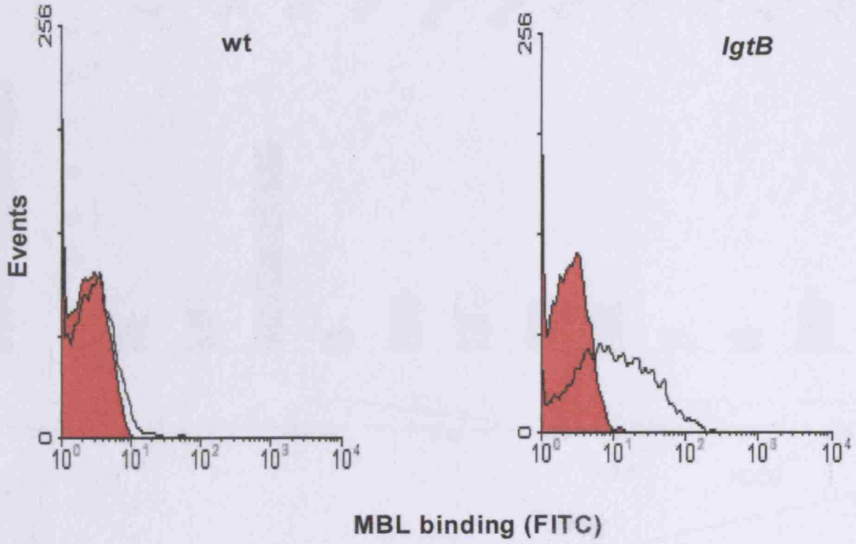


Figure 3-6. Representative flow cytometric profiles of MBL binding to the parent and *ltgB* mutant of *Neisseria meningitidis H44/76.*

MBL was added at a final concentration of 3.5 μg/ml. The control histogram (red) shows samples incubated with FITC anti-MBL alone, with the experimental samples ("uncoloured") in which samples have been incubated with MBL and then FITC anti-MBL. This experiment was repeated three times, and the profiles shown are representative.

Minimal binding of MBL was seen to eight of the nine *Neisseria* strains studied. The concentration of MBL used in these studies was based upon the median concentration of 3.2 μg/ml ascertained from the studies described in Chapter 4.

At the chosen concentration of 3.5 μg/ml, there was distinct MBL binding to the *ltgB* organisms ($P<0.05$). Typical profiles are shown in Figure 3-6. There was no significant binding to the parent H44/76 or other mutant organisms. MBL binding to all the LPS structures studied is summarised schematically in Figure 3-7.

MBL binding to the *ltgB* was dose dependent and even at concentrations of 6ug/ml there was no evidence of a plateau.

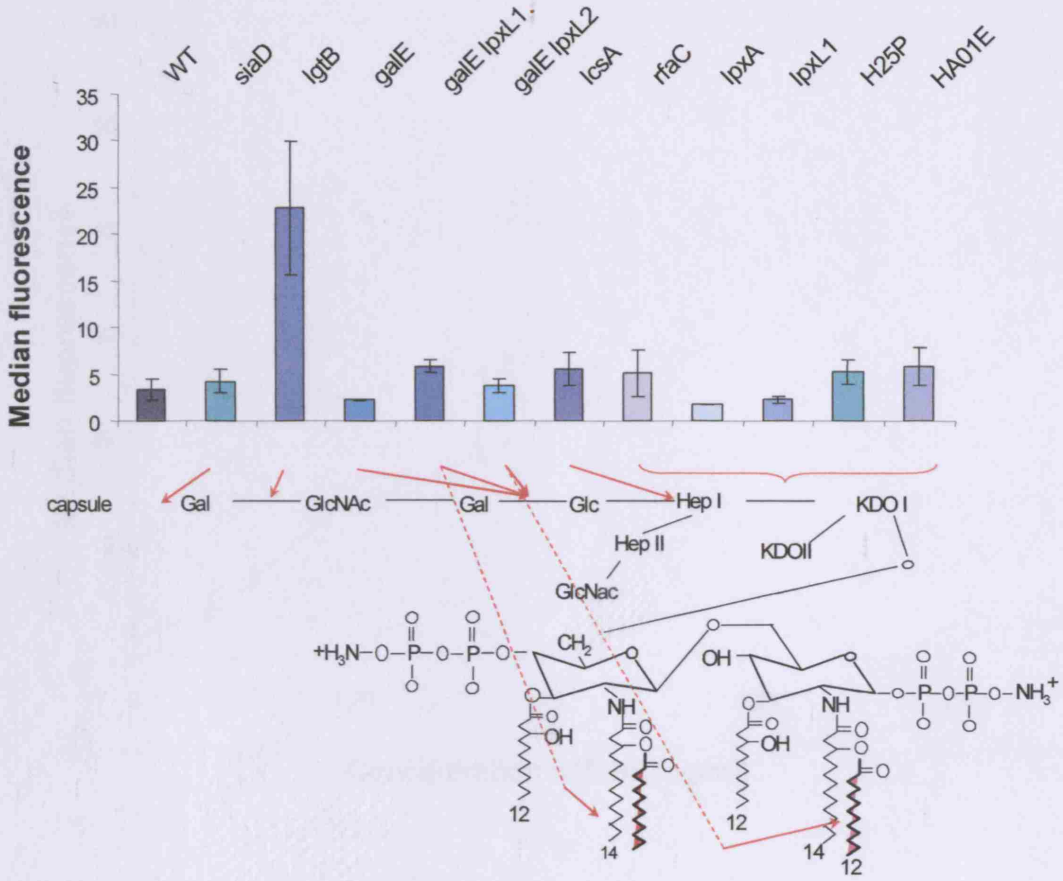


Figure 3-7. MBL binding to nine different *Neisseria meningitidis* H44/76 organisms and the corresponding LPS structure.

MBL was added at a final concentration of 3.5 μg/ml. The histogram shows samples incubated with MBL and then FITC anti-MBL. Each experiment was repeated at least three times. The error bars represent the upper 95% confidence interval. The lower panel shows the LPS structure of *Neisseria meningitidis H44/76*. The arrows show truncation positions of the LPS structure.

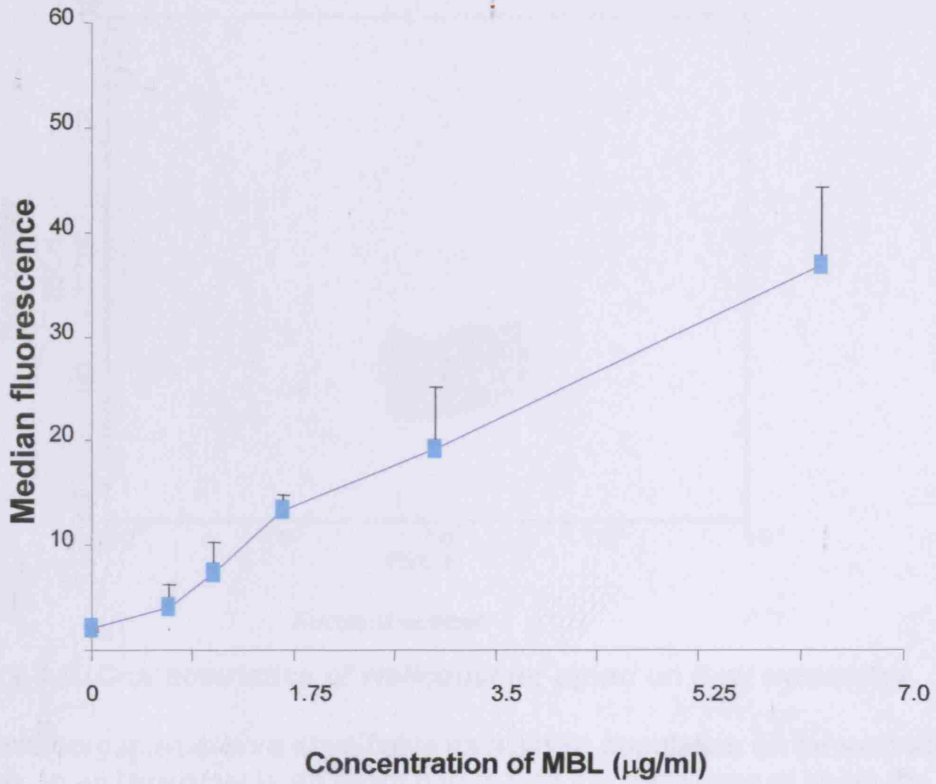


Figure 3-8. Dose-dependent MBL binding to *N.meningitidis H44/76 ltgB mutant.*

Binding of MBL is expressed as the mean of median fluorescence for MBL binding from 4 experiments. Error bars indicate upper 95 % confidence intervals.

3.3.1.2 MBL binding to *Helicobacter pylori*

Flow cytometry was also used to investigate the interaction of MBL with Helicobacter *pylori* NCTC11637 and two isogenic LPS mutants. As with *Neisseria* it was possible to detect a distinct population based on forward and side scatter. Events were therefore collected on the basis of size and granularity (Figure 3-9).

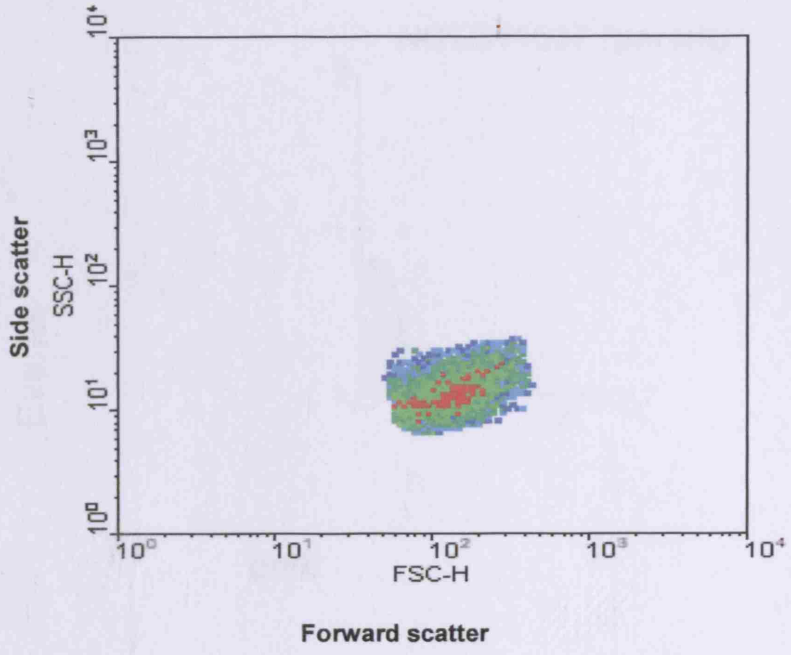


Figure 3-9. Characteristics of *Helicobacter pylori* on flow cytometry.

The microorganisms were identifiable as a single population on forward and side scatter. In all experiments an event had to fulfil the conditions of being the correct size and granularity.

There were marked differences in the binding of MBL to the three organisms studied. Typical profiles are shown in Figure 3-10. At a concentration of MBL equal to 3.5 μg/ml there was no detectable binding to the parent organism. However, the two mutant *Helicobacter pylori* bound MBL avidly. Binding was concentration dependent (Figure 3-11)

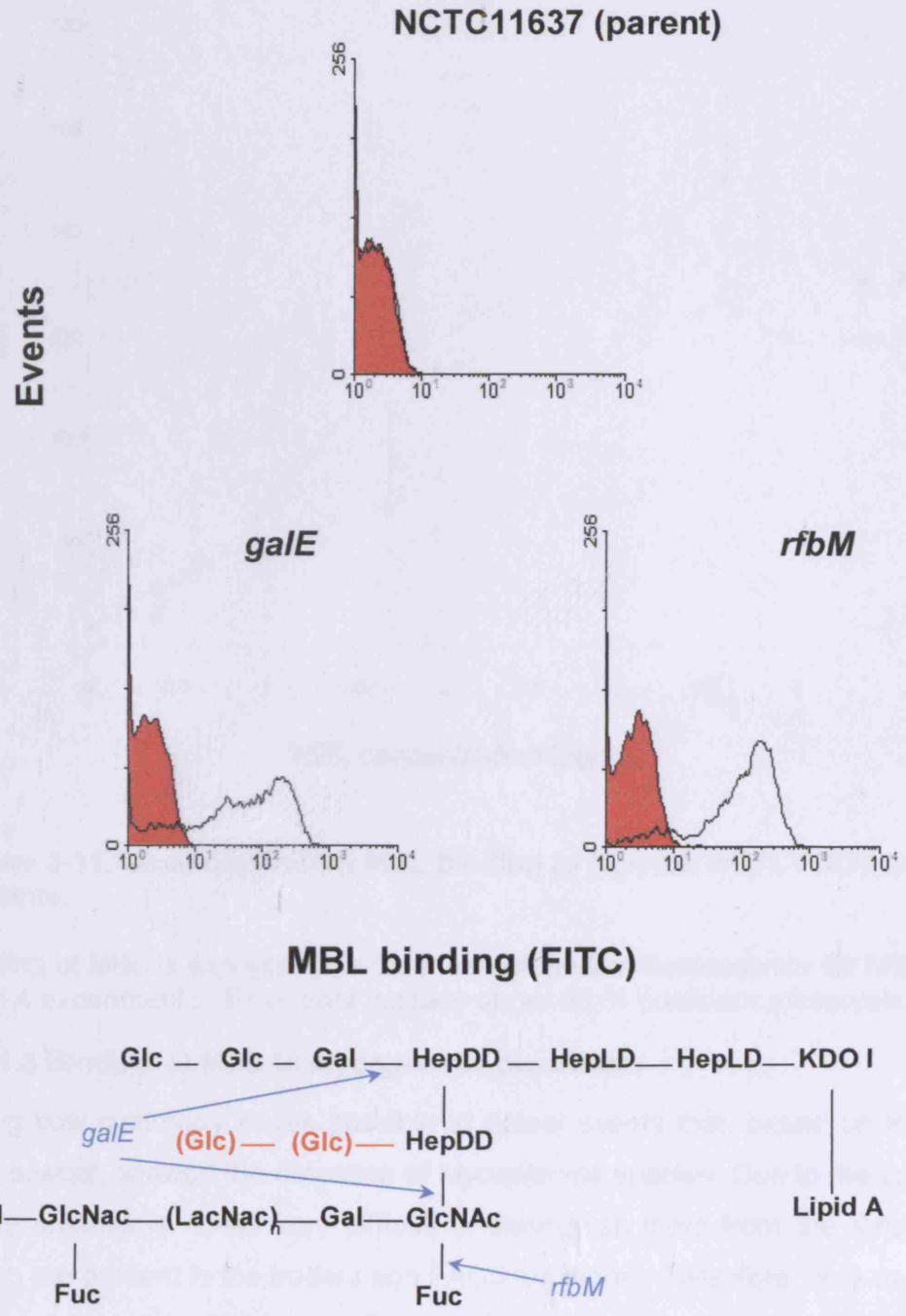


Figure 3-10. Representative flow cytometric profiles of MBL binding to the parent (NCTC11637) and two mutants of *H.pylori*.
MBL was added at a final concentration of 3.5 μg/ml. The control histogram (filled red) shows samples incubated with FITC anti-MBL alone, with the experimental samples (uncoloured) incubated with MBL and then FITC anti-MBL. This experiment was repeated five times for all organisms, and the profiles shown are representative. The lower panel shows the LOS structure for the NCTC11637 organism with the truncation points of *galE* and *rfbM* marked.

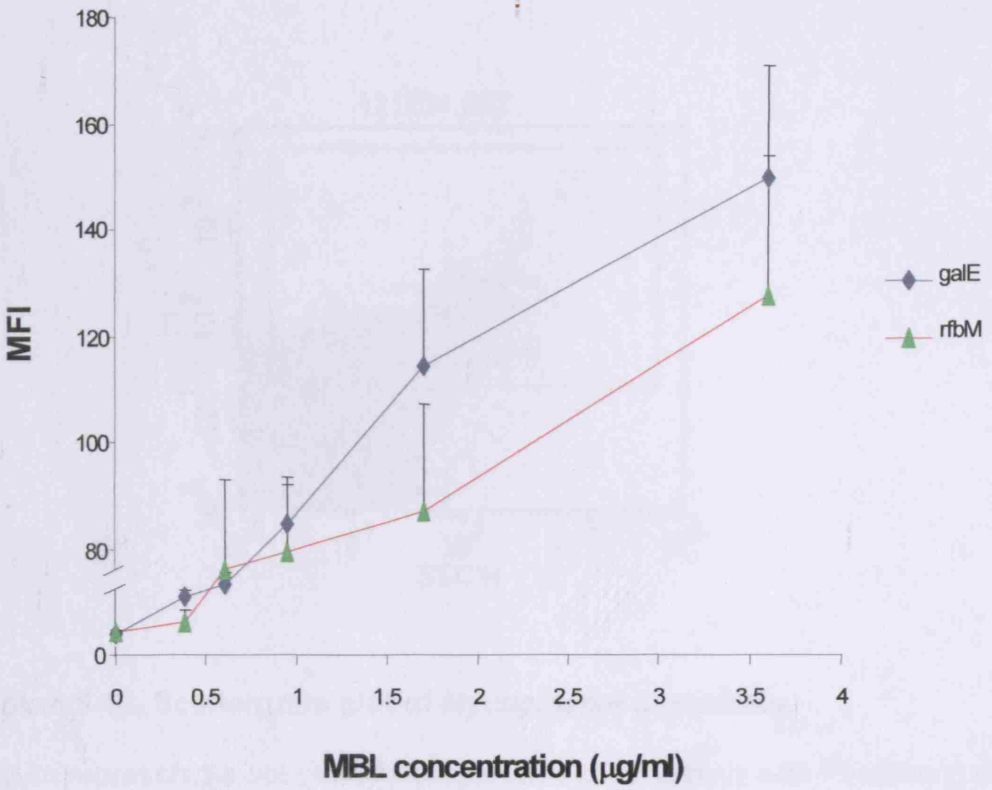


Figure 3-11. Dose-dependent MBL binding to *H.pylori NCTC11637* isogenic mutants.

Binding of MBL is expressed as the mean of median fluorescence for MBL binding from 4 experiments. Error bars indicate upper 95 % confidence intervals.

3.3.1.3 Binding of MBL to *Mycoplasma* organisms

Using flow cytometry it was possible to detect events that, based on forward and side scatter, allowed the detection of *Mycoplasma* species. Due to the small size of these organisms it was very difficult to distinguish them from the small particles which are present in the buffers and FACS solutions. Therefore, only events which were positive for propidium iodide staining, indicating presence of DNA or RNA (Figure 3-12), were analysed.

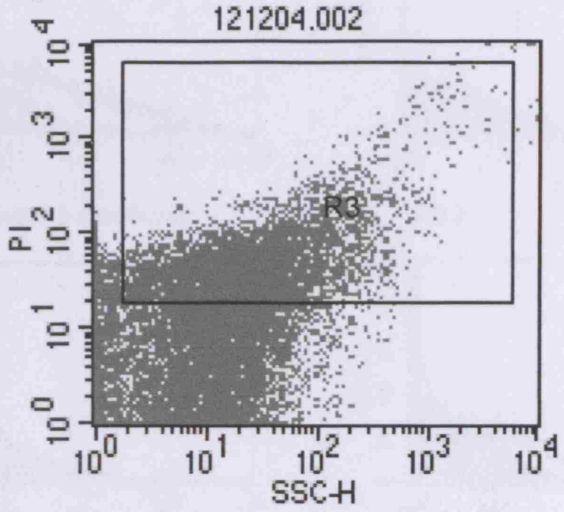


Figure 3-12. Scattergram plot of *Mycoplasma* organisms.

Figure represents a dot plot of a preparation of *M.hominis* with PI staining depicted on the y-axis and side scatter along the x-axis. The rectangular acquisition gate is shown.

There were marked differences in the binding of MBL to the four organisms studied. Typical profiles are shown in Figure 3-13. There was significant binding to *Mycoplasma hominis*, *Mycoplasma orale* and *Mycoplasma pneumoniae* ($p<0.05$, Kruskal-Wallis H-test). There was no binding to *Mycoplasma* A39.

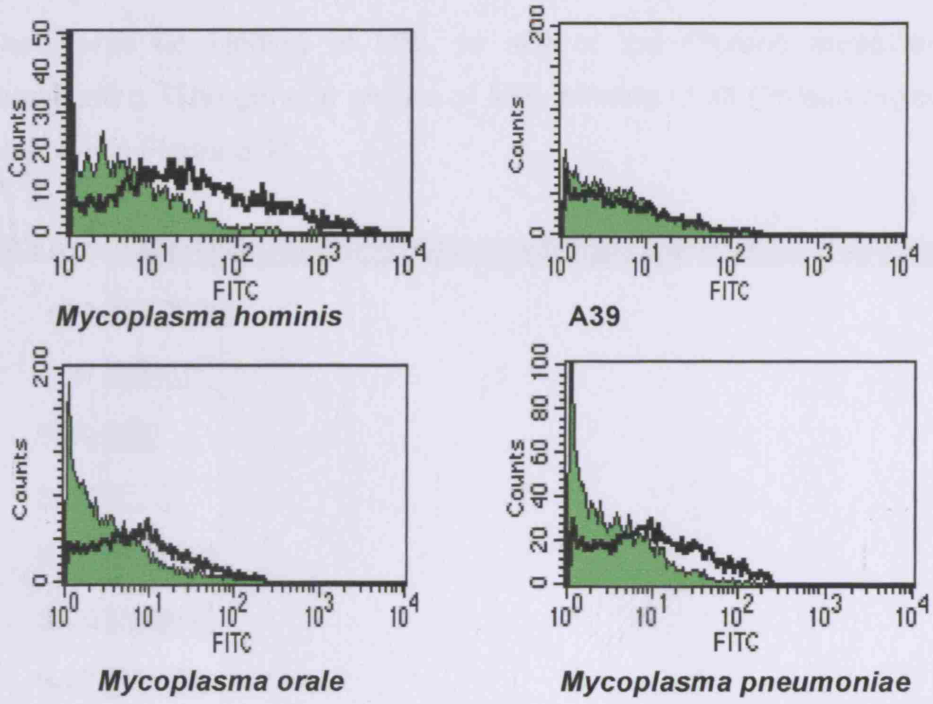


Figure 3-13. Representative flow cytometric profiles of MBL binding to different *Mycoplasma* organisms.

MBL was added at a final concentration of a 5 μg/ml. The control histogram (filled green) shows samples incubated with FITC anti-MBL alone, with the experimental samples (unfilled) incubated with MBL and then FITC anti-MBL. This experiment was repeated five times for all organisms, and the profiles shown are representative.

3.3.1.4 MBL binding to *Proteus mirabilis*

There was no binding of MBL to any of the *Proteus mirabilis* preparations investigated. The general picture of MBL binding to all *Proteus* organisms studied is shown in Figure 3-14.

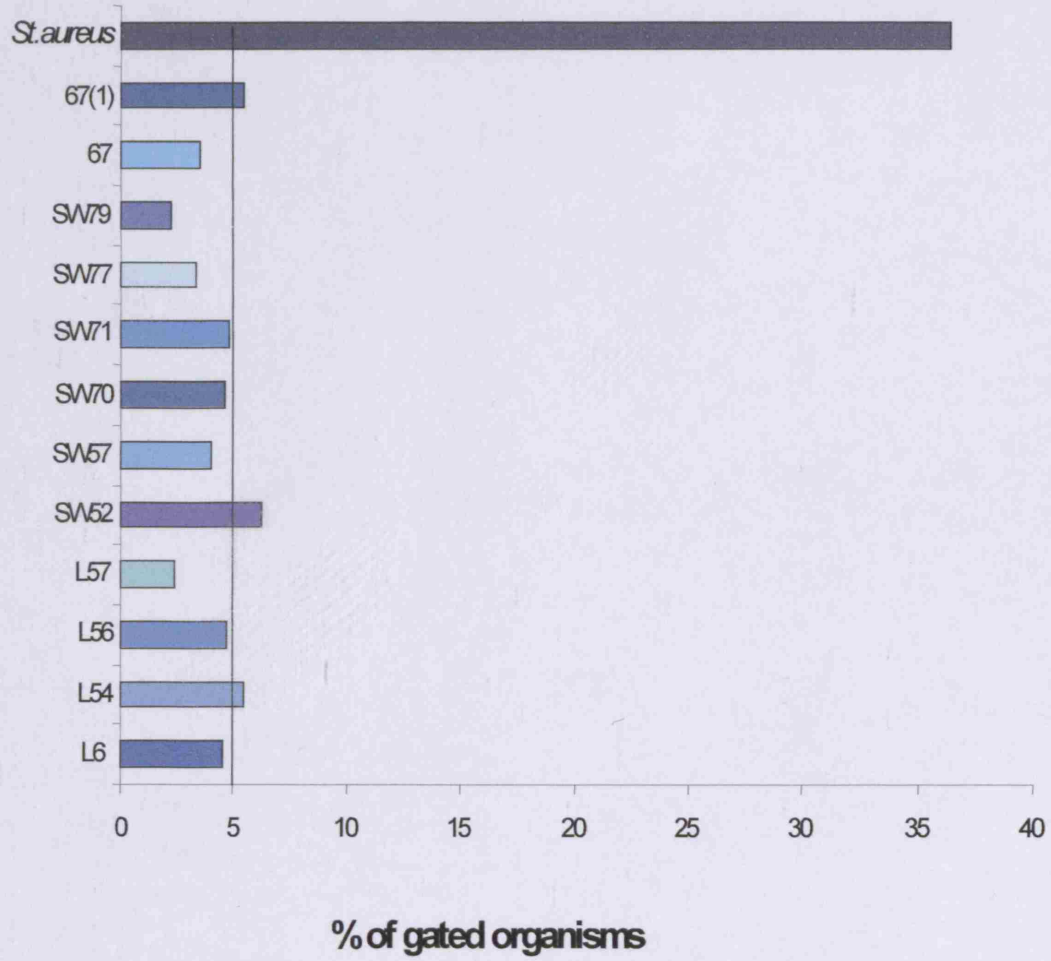


Figure 3-14. Absence of MBL binding to different strains of *Proteus mirabilis*.

MBL was added at a final concentration of 3.5 μg/ml. The histogram shows samples incubated with MBL and then FITC anti-MBL. Each experiment was repeated no less than three times. The error bars represent the upper 95% confidence interval. *S. aureus* was used as a positive control.

3.3.2. Specificity of MBL binding

In order to evaluate whether the MBL binding observed was mediated by C-type lectin interactions, inhibition experiments using different concentrations of monosaccharides (D-mannose, galactose and *N*-acetyl-D-glucosamine) as well as the calcium chelating agent EDTA were performed. The monosaccharide or EDTA was added to the MBL solution 10 min prior to the addition of the MBL to the six different microorganisms that bound MBL. These were *Neisseria meningitidis ltgB, the Helicobacter* species *galE and rfbM* and the three *Mycoplasma* species, *hominis, orale, pneumonia*.

The binding of MBL to all organisms was inhibited by the monosaccharides mannose and N-acetylglucosamine as well as by the addition of EDTA. As expected for a C type lectin interaction galactose did not inhibit MBL binding to any of the six organisms.

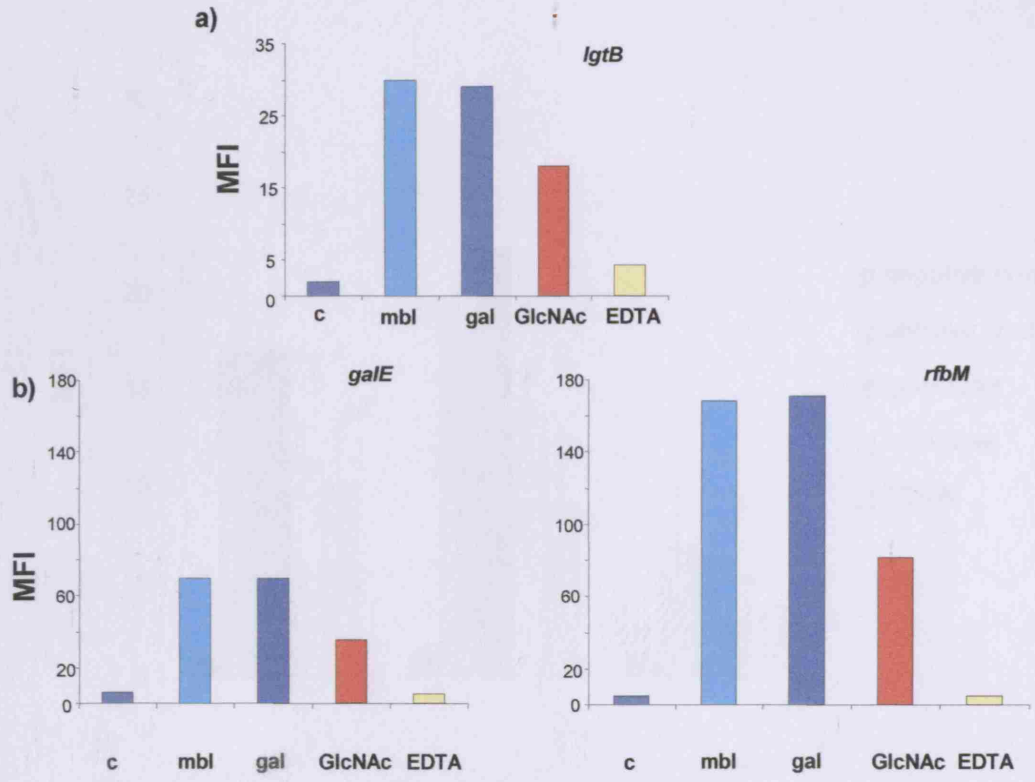


Figure 3-15. Effect of different monosaccharides and EDTA on the binding of MBL to the selected organisms.

Panel (a) shows the effect of galactose, N-acetyl-glucosamine and EDTA on MBL binding to ltgB mutant of *Neisseria meningitidis* 44/76, while panel (b) represents the results from similar experiment with two mutants of *Helicobacter pylori*. The monosaccharide (galactose at 25 mM concentration, GlcNAc at 25 mM concentration) or EDTA (at 5 mM concentration) was added to the MBL solution 10 minutes prior to the addition of the MBL to the organisms. MBL was added at a concentration of 3500 ng/ml. The binding of each monosaccharide or EDTA was expressed as a representative median fluorescence of MBL binding to the *galE* and *rfbM* mutants. The figure shows a representative example of three separate experiments.

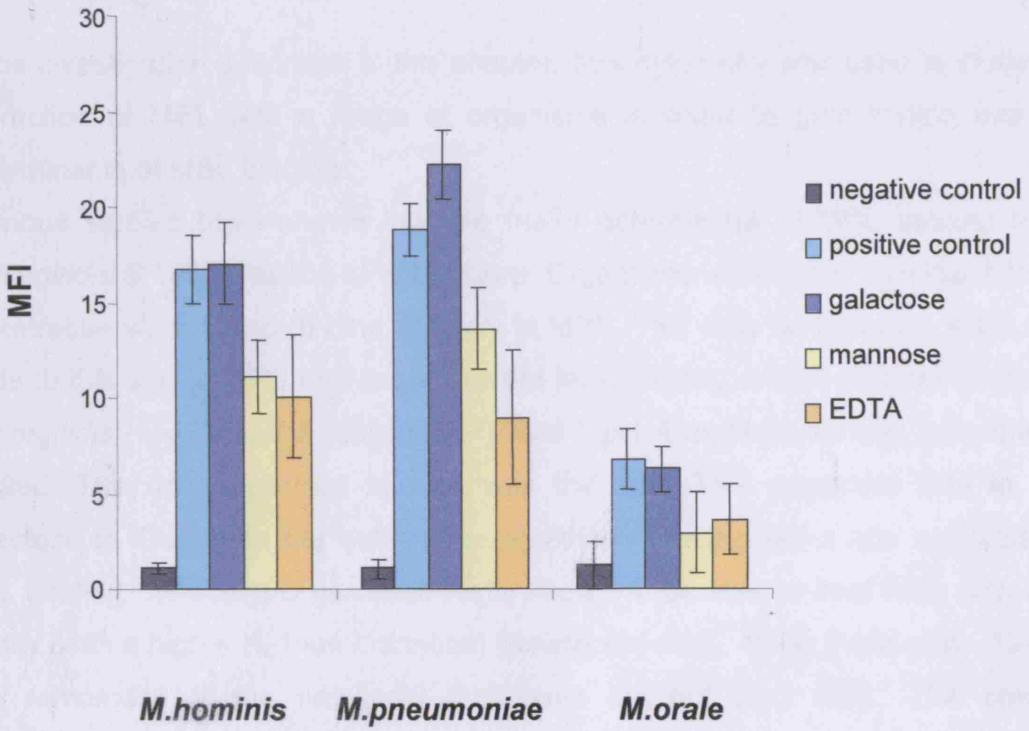


Figure 3-16. Effect of different monosaccharides and EDTA on the binding of MBL to *Mycoplasma* organisms.

The monosaccharide (galactose at 25 mM concentration, mannose at 25 mM concentration) or EDTA (at 5 mM concentration) was added to the MBL solution 10 minutes prior to the addition of the MBL to the organisms. MBL was added at concentration 5000 ng/ml. The binding of each monosaccharide or EDTA was expressed as a representative median fluorescence of MBL binding. The figure shows a median of three separate experiments ±SD.

3.5. Discussion

In the investigation described in this chapter, flow cytometry was used to study the interaction of MBL with a range of organisms in order to gain insight into the determinants of MBL binding

Previous studies have shown that the major determinant of MBL binding to *N. meningitidis B* 1940 was the LPS structure. Organisms lacking the terminal n-lacto-neotetraose were found to bind strongly to MBL. This may be because sialic acid binds to this site on LPS and might prevent MBL binding. In this chapter *Neisseria meningitidis H44/76* and a range of LPS and Lipid A mutants derived from it were studied. The only organism to bind was the *ltgB*. This organism fails to add galactose to GlcNac in the outer core, potentially making this sugar available for MBL binding. N-acetyl-D-glucosamine is known to be able to bind MBL with high affinity (with a higher K_D than mannose) (Kawakami *et al.*, 1984; Ihara *et al.*, 1991). The remainder of the neisserial organisms did not bind MBL. The smooth chemotype organisms 44/76 and *siaD*, both have a complete LPS structure terminating with galactose. However the two organisms differ because the *siaD* lacks a capsule. This finding therefore supports previous studies which have shown that capsule and a full length neisserial LPS prevent lectin binding to this organism. Interestingly, this pattern of MBL binding may be similar for other gram negative organisms such as *Salmonella* species (Devyatyarova-Johnson *et al.*, 2000).

There was also no MBL binding to the *galE-* organism. This was less predictable as a similar *galE-* mutant of *Neisseria meningitidis* serogroup B (B1940 *cps-*) bound MBL strongly, even at low collectin concentrations. The only difference between B1940 and 44/76 is that glucose on the beta chain in B1940 had been replaced by phosphethanolamine in 44/76. Therefore it is possible that MBL is able to bind to this glucose residue. Alternatively MBL could be prevented from binding to the terminal glucose on the α-saccharide chain because of steric changes in the 44/76 as a result of this substitution.

The LPS of the *icsA* mutant contains 2 molecules of ADP-L-glycero-D-manno-heptose attached to the KDO1-lipid which, at least for mouse MBL, has been

shown to be a major determinant of binding (Kawakami *et al.*, 1984; Ihara *et al.*, 1991). Furthermore the heptose II has a terminal N-acetylglucosamine which should be a potential site for MBL binding. The L-D optical form of heptose I appear to bind MBL to *Salmonella typhimurium* and *Neisseria gonorrhoeae*. Therefore it is likely that the presence of the phosphethanolamine in the beta side chain is somehow able to inhibit MBL binding in the 44/76.

Heptose-deficient mutant *rfaK*, which failed to add heptose to KDO1-lipid A, shows no MBL binding and these data are in full agreement with our previous findings (Devyatyarova-Johnson *et al.*, 2000).

The variations in lipid A structure in all organisms studied did not influence the MBL binding pattern.

Taken together the results presented in this chapter support the view that LPS structure and/or composition is important in determining MBL binding to Neisseria (Jack *et al.*, 1998; Devyatyarova-Johnson *et al.,* 2000). What is not known, however, is how much MBL has to bind to have a physiological effect. Using more sensitive methods such as electron microscopy with immunogold detection of bound MBL, suggested that low level binding could be physiologically relevant (Jack *et al.*, 2001). Similar findings have been reported for *Pseudomonas aeruginosa* (Møller-Kristensen *et al.*, 2006) in which FACS analysis failed to detect MBL binding that was observed with more sensitive methods.

The next organism to be investigated was *Helicobacter pylori NCTC11637* and its two isogenic mutants. The parent organism possesses an intact LPS, and did not bind MBL at any concentration. The O-specific chain of the *H.pylori* LPS mimics Lewis blood group antigens in structure (Edwards *et al.,* 2000) and therefore it would be surprising if MBL bound to this strain.

Structural analysis of the LPS synthesized by the *galE* mutant reveals the addition of an α-1-6 linked glycan chain, not present in S-form LPS, and an incomplete Le^x unit, comprising Fuc-(1-3)-GlcNAc-(1-7)- to the fourth heptose unit in the inner core, forming a branched structure (Edwards *et al.*, 2000). Therefore there are

many available binding sites for MBL. This is not obviously the situation with the *rfbM* mutant in which the LPS structure was indistinguishable from that of wild-type *H.pylori* apart from a lack of GDP-fucose (Edwards *et al.*, 2000). The LPS expressed by this strain has a modified O-antigen side chain that no longer contains structures homologous to Le^x. It seems that the presence of Le^x type structures on the surface of *Helicobacter* prevents MBL binding, whereas the lack of these carbohydrates dramatically increases lectin binding. This again indicates the importance of LPS structure in determining the capacity of MBL to bind to gram-negative organisms.

There are no data published on the role of MBL in *H.pylori* infections, probably because MBL would not be expected to exist at high concentrations within the GI tract. However in inflammatory conditions, MBL can be found as a transudate (Garred *et al.*, 1993; Malhotra *et al.* 1995). MBL had been detected in duodenal fluid (Kelly *et al.*, 2000). The data in this chapter indicates that some structural forms of this organism could potentially be targets of MBL if it were present within the GI tract.

The collectin SP-D, which is detected within the gastric mucosa, does appear to be important in this infection. *Helicobacter pylori* up-regulates the expression of SP-D in human patients with gastritis and in SP-D deficient (SP-D (-/-)) mice colonization with *Helicobacter* is more common. SP-D binds and agglutinates *H.pylori cells* in a lectin-specific manner (Khamri *et al.,* 2005), and has been shown to bind *H.pylori* lipopolysaccharides (Moran *et al.*, 2005).

The expression of *H.pylori* O antigen is subject to phase variation (Appelmelk *et al.*, 1998) as in *Neisseria* species and in this organism the monosaccharide units of O antigen are transferred sequentially, rather than as a complete unit. It is possible that MBL and other collectins could bind to incomplete LPS structures and thereby help to eliminate these species during their development.

Clinical isolates of *Mycoplasma pneumoniae*, *hominis* and *orale* bound MBL in a Ca^{2+} -dependent and sugars specific manner characteristic of a C-type lectin. This binding may explain the findings reported in the study of patients with primary antibody deficiency (PAD) (Hamvas *et al.*, 2005) in which MBL deficiency was found to influence the susceptibility to mycoplasma infections. A recent study

(Piboonpocanun *et al.*, 2005) showed that preparations of human and rat surfactant-A could bind to the lipid extracts prepared from *Mycoplasma pneumoniae* with high affinity in the presence of Ca^{2+}. The major lipid ligands for the protein, identified by mass spectrometry, were a group of di-saturated phosphatidylglycerols. SP-D also binds intact *Mycoplasma* and membranes with high affinity (Chiba *et al.*, 2003). The potential determinant for MBL binding on *Mycoplasma* is yet to be established. It is likely that there are mannose residues on the surface of mycoplasmas, but this needs to be formally explored.

Several studies of patients suffering with rheumatoid arthritis (RA) have documented the presence of elevated levels of serum antibodies to *Proteus mirabilis* suggesting a role for this organism in this disease (Senior *et al.*, 1995; Blankerberg-Sprenkels *et al.*, 1998; Chou *et al.*, 1998; Wanchu *et al.*, 1997; Subair *et al.*, 1995). A number of studies have also implicated MBL as a modulator of disease severity (Graudal *et al.*, 2000; Saevarsdottir *et al.*, 2001; Horiuchi *et al.*, 2000; Stanworth *et al.*, 1998; Kilpatrick *et al.*, 1997). It was with this in mind that the binding to *Proteus* was investigated. There was no MBL binding to any *Proteus mirabilis* organisms studied. We do not know the exact LPS structure of the *Proteus* species used, but the O-polysaccharide of published strains of *Proteus mirabilis* shows the presence of a tetrasaccharide repeating unit rich in galactose and GalNac (Zablotni *et al.*, 2005). These sugars do not bind MBL and, once again, LPS structure is shown to be important in determining MBL binding to a bacterium.

The results from this chapter confirm that LPS structure is an important determinant of MBL binding. However, the ability to predict MBL binding on the basis of terminal sugars alone is unreliable. LPS folding, density, and other factors influencing steric conformation on the bacteria are also important and will determine whether MBL might have a possible protective role in human pathology.

Chapter 4 MBL: studies of an unselected population

4.1 Introduction

Prior to the availability of practical genotyping methods, MBL studies relied upon protein measurements (Super *et al.*, 1989; Terai *et al.*, 1993; Lu *et al.*, 1990; Sumiya *et al.*, 1991; Ezekowitz *et al.*, 1988, 1989). A range of different assays have been used to measure MBL protein. These assays include measurements of MBL peptide levels using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Garred *et al.*, 1992; Thiel *et al.*, 1992), measurement of MBL binding to mannan in mannan-binding assays (Holmskov *et al.*, 1993; Thiel *et al.*, 1992), radioimmunoassay (Kawasaki *et al.*, 1989), and time-resolved immunofluorometric assay (TRIFMA) (Christiansen *et al.*, 1999). There were big discrepancies in MBL levels measured by different groups. The median MBL level measured by mannan-binding assay using rabbit anti-MBL antibodies in ten children with the opsonic defect was 4.9 μg/l compared to 143 μg/l in a control group (Super *et al.*, 1989). The measurement of MBL concentration in 103 blood donors by the same method demonstrated that protein levels ranged from 0 to 870 μg/l. The mean MBL level measured by radioimmunoassay was reported as 1.82 μg/ml (Kawasaki *et al.*, 1989). Another study using ELISA reported that levels of MBL, determined for a total of 1085 normal sera, ranged from 0.07 to 6.40 μg/ml (Terai *et al.*, 1993). These serum MBL values were 10 times higher than MBL levels reported by other groups (Lu *et al.*, 1990; Super *et al.*, 1989; Sumiya *et al.*, 1991), 10 times lower than MBL levels reported by Ezekowitz *et al.* (1988, 1989), and compatible with MBL levels quoted by Kawasaki *et al.* (1989).

Corrected data on UK MBL levels which agreed with data for Japanese individuals, published by Terai *et al.* (1993), was reported (Super *et al.*,1992). Thiel *et al.* (1995) established that their previously published MBL levels were underestimated by a factor of approximately 10. In ten laboratory members MBL levels ranged from 58 to 2916 ng/ml. Similarly, the concentration of MBL in 123 Danish blood donors showed a wide range of MBL levels from 0 to 4889 μg/l. Relatively few studies have compared different methods on identical samples (Minchinton *et al.*, 2002).

Since the discovery of MBL polymorphisms, a number of studies have been performed in which both genotype and phenotype have been recorded. (Garred *et*

al., 1992; Lipscombe *et al.*, 1992; Madsen *et al.*, 1994, 1995, 1998; Steffensen *et al.*, 2000; Minchinton *et al.*, 2002; Ip *et al.*, 2004).

However, few large studies, either in the UK or elsewhere, have been performed in which genotype and phenotype have been measured in healthy individuals. This chapter describes an extensive population based study to determine both MBL genotype frequencies and genotype-phenotype correlations.

Acknowledgments

I am grateful to Dal Dhillon (Dade Behring UK) and Janine Rolland (Clinical Immunology Department, GOSH) for performing immunonephelometric assays.

The genotype data for the control cohort of unselected population had been provided by ALSPAC group (Bristol and Institute of Child Health, London).

4.2 Materials and Methods

4.2.1 Samples

The AVON Longitudinal Study of Pregnancy and Childhood is a part of a European-wide World Health Organisation prospective study to determine factors influencing the health and development of children in Europe. Some 13,995 expectant mothers in the Bristol area were recruited into the study, and finally 14,138 children were involved.

A random subset of the main study group, comprising approximately 1,400 children, was selected for more in-depth analysis of health factors. This group was called the Children in Focus series, and each member of the group had blood taken for DNA extraction and plasma isolation for measurement of the MBL level.

4.2.2 Measurement of plasma MBL concentration

The ELISA method described in Section 2.4.6 and 2.5, pages 13-14 was used to determine the MBL levels in serum samples. MBL Oligomer ELISA kit from AntibodyShop (Copenhagen, Denmark) was used in this study and compared with an in-house procedure. Results from assays were included if the intermediate standard concentration on the ELISA plate was within the accuracy limits of the assay. The immunonephelometric assays were performed on a BN (Behring) II nephelometer housed and maintained in the Clinical Immunology laboratories of Great Ormond Street Hospital for Children, London. In order to standardise (minimise variation) both assays were performed on the same day.

4.2.3 MBL genotyping

The heteroduplex genotyping method described in Section 2. 7.3 and 2.7.4, pages 16-17 was used to genotype both structural region and promoter region polymorphisms of the MBL2 gene of the samples.

4.2.4 Statistical analysis

The Kruskal-Wallis nonparametric test was used in this study in order to determine differences in MBL levels in different groups.

4.3 Results

4.3.1 Agreement between two different types of ELISA procedures

In a study of 51 clinical paediatric serum samples there was a good correlation between the two ELISA procedures (r^2=0.9166, p<0.0001). However, such analyses can be misleading and underestimate the differences between the assays (Fig 4-1). Another statistical approach has therefore also been applied to these data. The plot of difference against mean (Figure 4-3) shows that the MBL concentration determined by in-house ELISA can be as much as 1346 ng/ml above or 1361 ng/ml below the MBL level determined by MBL Oligomer ELISA kit. Therefore, these two assays gave markedly different readings.

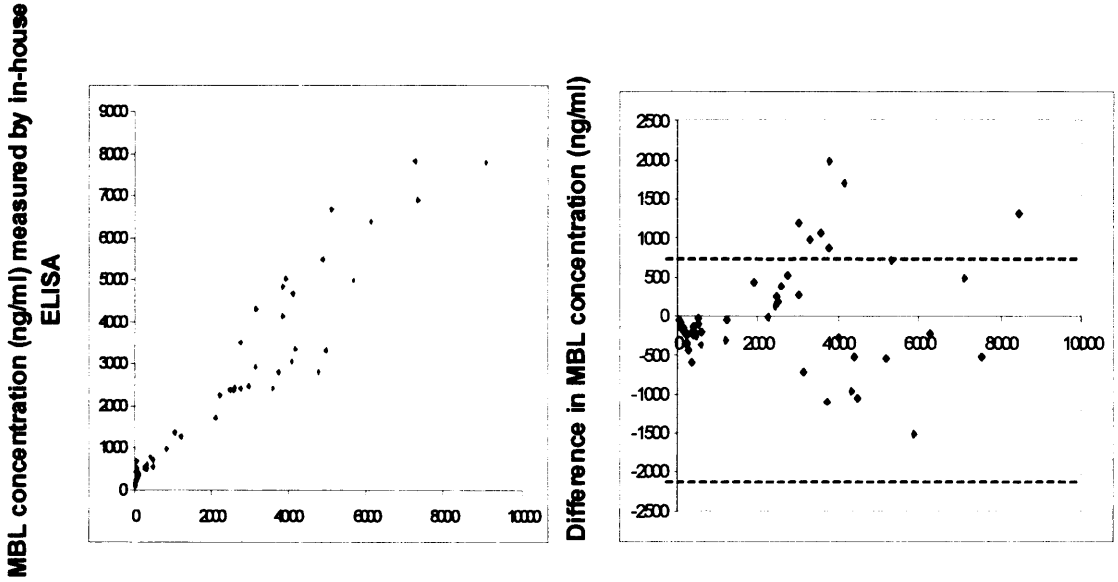


Figure 4-1. The correlation and agreement between two ELISA methods for measuring MBL concentration.

4.3.2 Comparison between AntibodyShop MBL Oligomer ELISA and immunonephelometry for determining MBL levels in serum

In a separate study of 54 clinical paediatric samples (mostly from patients with frequent infections), a poor correlation was noted between the AntibodyShop ELISA and the Behring immunonephelometric assay system (r^2=0.6006). Using the ELISA procedure, 27 of these samples were found to have MBL levels below 1000

ng/ml and in 20 samples the levels were < 500 ng/ml. In contrast, using the immunonephelometric procedure, only three samples were found to have levels below 1000 ng/ml with the lowest level being 710 ng/ml.

4.3.3 Comparison between Genotype, AntibodyShop ELISA and Immunonephelometry

To determine the relationship between MBL genotype and phenotype, MBL levels were measured by AntibodyShop ELISA and immunonephelometry and correlated with MBL genotypes in a cohort of 32 children with Cystic Fibrosis using the method described in section 2.7.3 and 2.7.4. The data obtained are summarised in the Table 4-1.

Sample	ELISA (ng/ml)	Nephelometry (ng/ml)	Short MBL haplotype	Expected MBL phenotype
1	<50	1760	YO/YO	Low
2	709	2420	XA/YO	
3	<50	1590	XA/YO	
4	<50	802	XA/YO	
5	180	1570	YA/YO	
6	<50	768	YA/YO	
7	<50	1530	YA/YO	
8	244	1490	YA/YO	
9	702	1680	YA/YO	
10	277	2240	YA/YO	
11	401	1620	YA/YO	
12	1877	1730	YA/YA	Normal
13	2119	3600	YA/YA	
14	3792	5150	YA/YA	
15	5555	6750	YA/YA	
16	4876	5170	YA/YA	
17	2201	2900	YA/YA	
18	3456	3650	YA/YA	
19	3590	2220	YA/YA	
20	878	2150	YA/YA	
21	738	2190	XA/YA	
22	4541	4280	XA/YA	
23	4193	4000	XA/YA	
24	3874	2200	XA/YA	
25	2148	2010	XA/YA	
26	2546	1880	XA/YA	
27	1957	2490	XA/YA	
28	2059	2370	XA/YA	
29	3803	3850	XA/YA	
30	2250	3300	XA/YA	
31	1299	2540	XA/YA	
32	2931	2740	XA/YA	

Table 4-1. MBL levels in a cohort of children with cystic fibrosis measured by AntibodyShop ELISA and immunonephelometry.

A reasonable correlation exists between the two methodologies for individuals with wild-type alleles and an expected 'normal' MBL level. In contrast, the two methods were markedly discrepant when samples from individuals with MBL genotypes associated with low MBL levels were analysed. The plot of difference against the

mean in Figure 4-2 confirms the lack of agreement between two methods. The immunonephelometric MBL concentration ranged 831 ng/ml below to 5871 ng/ml above the MBL concentration determined by ELISA. Both techniques were performed according to the manufacturer's recommendations.

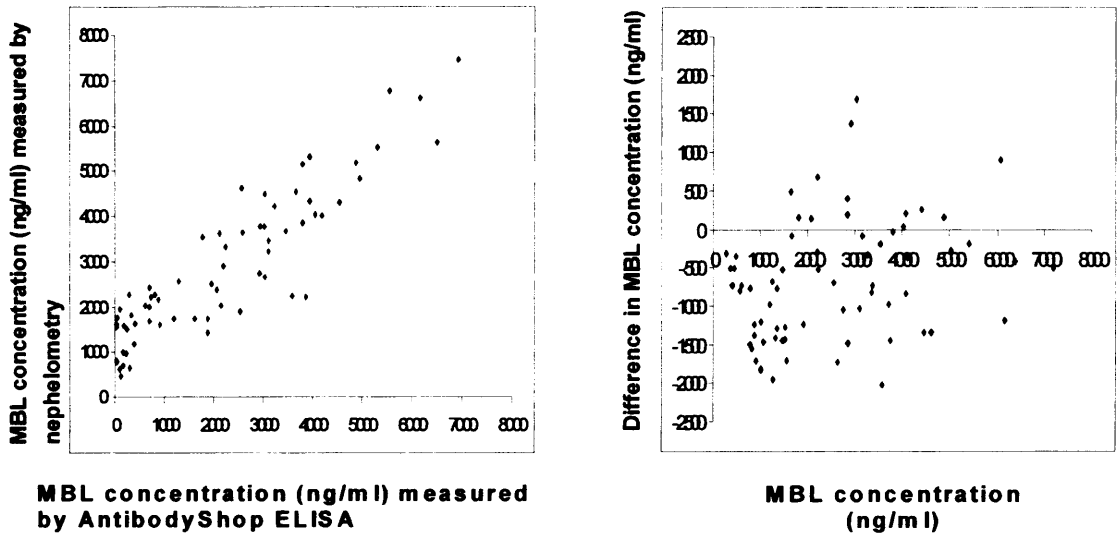


Figure 4-2. The correlation and agreement between immunonephelometry method and ELISA for measuring concentration of MBL.

4.3.3 Analysis of the relationship between MBL genotype and levels in children from the ALSPAC cohort

4.3.3.1 Genotype

Genotype and haplotype frequencies for a subsection of children in the ALSPAC Children in Focus population are presented in Table 4-2. Allele frequencies were in Hardy-Weinberg equilibrium and were consistent with previously published frequencies in Caucasian populations (Madsen *et al.*, 1995; Mead, 1997).

	Frequency	Percentage
LXPA/LXPA	26	3.9
LXPA/LYPA	16	2.4
LXPA/HYPA	72	10.9
LXPA/LYQA	53	8.0
LYPA/HYPA	17	2.6
LYPA/LYQA	7	1.1
HYPA/HYPA	53	8.0
HYPA/LYQA	75	11.3
LYQA/LYQA	24	3.6
LXPA/LYPB	28	4.2
LYPA/LYPB	3	0.5
LYPB/HYPA	33	5.0
LYPB/LYQA	25	3.8
LXPA/HYPD	18	2.7
LYPA/HYPD	7	1.1
HYPA/HYPD	16	2.4
HYPD/LYQA	17	2.6
LXPA/LYQC	3	0.5
LYPA/LYQC	1	0.2
HYPA/LYQC	4	0.6
LYQA/LYQC	2	0.3
LYPB/LYPB	15	2.3
LYPB/HYPD	10	1.5
HYPD/HYPD	5	0.8
HYPD/LYQC	2	0.3
Total	535	100

Table 4-2 MBL haplotype frequencies in Children in Focus cohort.

4.3.3.2 Plasma MBL levels

MBL levels can be seen to be broadly segregated into three groups (Figure 4-3), high, medium and low. The mean MBL level for the entire cohort was 2861.521 ng/ml (SD=1847, median = 3099 ng/ml), as measured by the AntibodyShop ELISA.

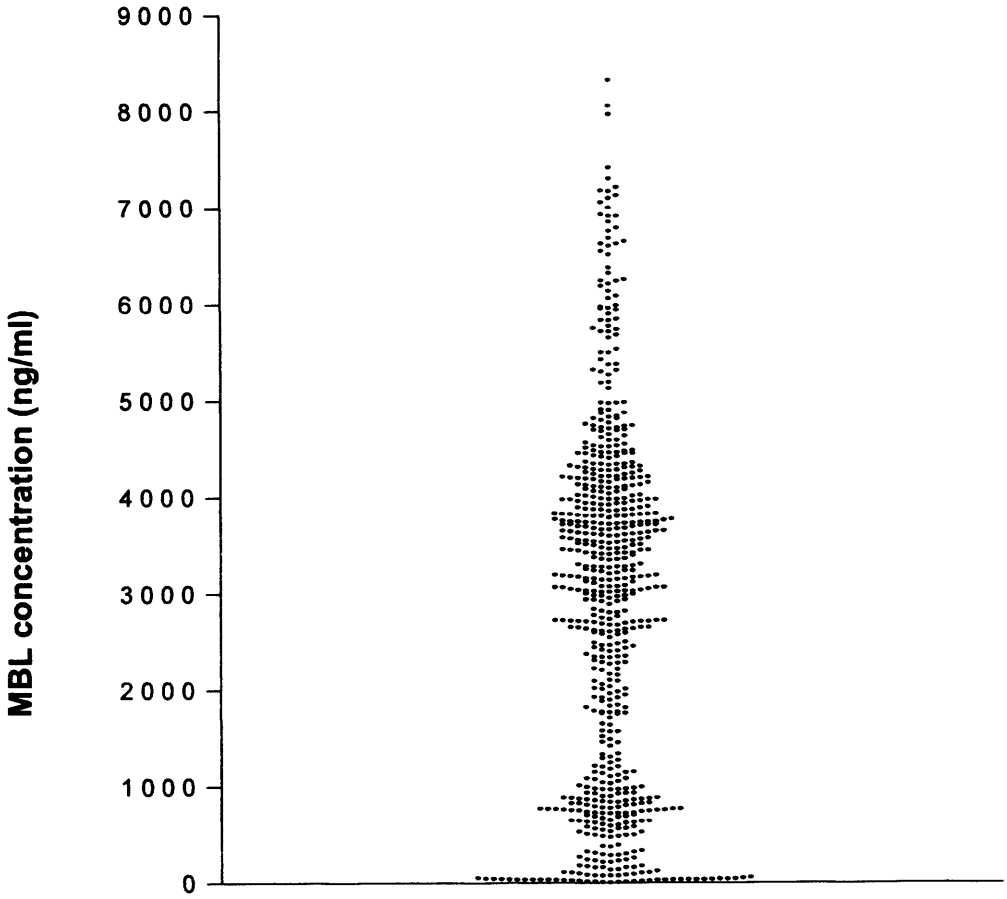
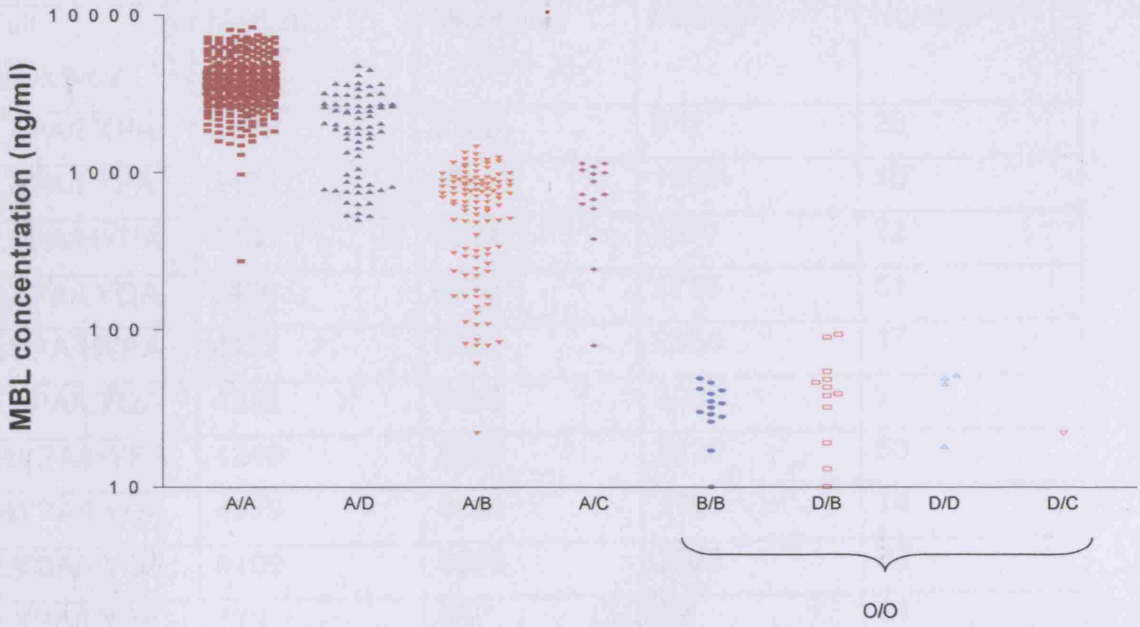


Figure 4-3. MBL concentrations in plasma samples from the Children in focus subgroup of the ALSPAC cohort.
Each dot represents an individual.

4.3.3.3 Plasma MBL levels stratified according to the MBL2 coding mutations

Figure 4-4 illustrates the range of MBL concentrations according to their exon 1 genotype. The mean MBL level, as determined by AntibodyShop ELISA, in 416 samples with wild type genotypes (AA), was 3928 ng/ml (SD=1281; Median=3773 ng/ml).



n	416	70	106	15	31
Mean	3928	2048	638	751	38
SD	1281	1083	358	329	19
Median	3773	1988	698	723	38

Figure 4-4. Mannose-binding lectin levels associated with various MBL2 exon1 genotypes.

Log_{10}[MBL] plasma is displayed against MBL exon 1 polymorphisms. As expected there was a clear relationship between Log_{10}[MBL]plasma and genotype (Kruskal-Wallis $p<0.0001$).

Despite these significant differences, there was some overlap between the MBL levels from the A/A and A/O groups such that some A/D and A/B individuals had MBL levels comparable with A/A children.

4.3.3.4 The distribution of plasma MBL concentrations in relation to the full MBL2 haplotype

The distribution of the complete genotypes with the corresponding means and ranges of MBL concentrations is shown in Table 4-3.

Full haplotype	Median	Maximum	Minimum	Number
LXPA/LXPA	2589	5189	272	26
LXPA/LYPA	3426	5841	1903	15
LXPA/HYPA	3768	6924	1577	72
LXPA/LYQA	3466	6679	1715	51
LYPA/HYPA	4197	6596	2355	17
LYPA/LYQA	4332	6183	2095	7
HYPA/HYPA	4249	8056	2226	53
HYPA/LYQA	4079	8324	1701	74
LYQA/LYQA	4109	6908	3522	22
LXPA/LYPB	179	857	22	31
LYPA/LYPB	569	881	498	3
HYPA/LYPB	836	1332	315	32
LYQA/LYPB	768	1455	495	25
LXPA/HYPD	778	1778	499	18
LYPA/HYPD	1779	2708	385	7
HYPA/HYPD	2682	4486	1925	18
LYQA/HYPD	2023	4757	1420	15
LXPA/LYQC	241	375	79	3
HYPA/LYQC	953	1084	586	4
LYQA/LYQC	858	995	722	2
LYPB/LYPB	32	49	10	15
LYPB/HYPD	41	93	13	12
HYPD/HYPD	47	56	18	6

Table 4-3. MBL plasma levels and corresponding haplotypes.

The correlation between MBL haplotypes and MBL concentrations is shown in Figure 4-5. HY haplotypes were associated with the highest level of MBL, LY haplotypes with a slightly lower level and LX haplotypes with the lowest levels of MBL in plasma. As shown in Table 4-3, the medians of the MBL concentration in

HYA homozygous individuals, LYA homozygous individuals and LXA homozygous individuals were 4249, 4220 and 2589 ng/ml, respectively.

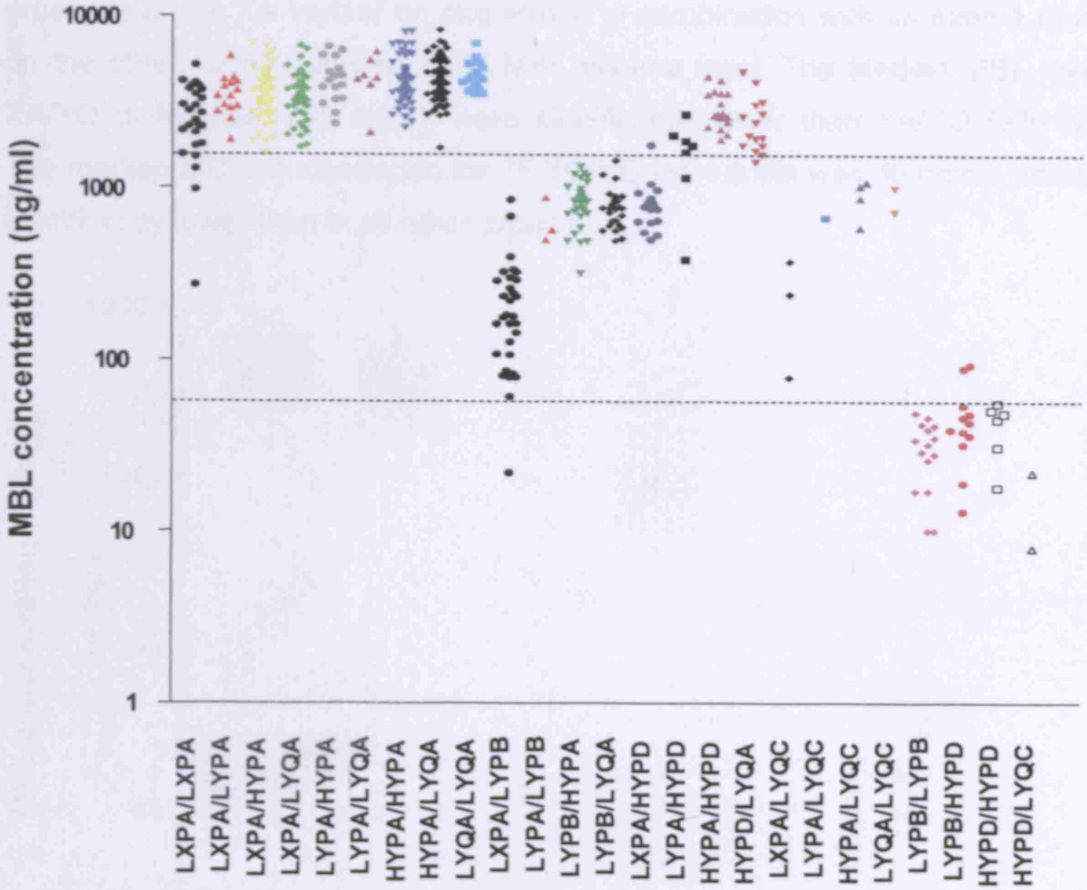


Figure 4-5. The MBL plasma concentrations and the corresponding complete MBL genotypes of children in the ALSPAC cohort.

Log_{10}[MBL] plasma is displayed against MBL haplotype (Exon 1 and full promoter polymorphisms). As expected there was a clear relationship between Log_{10}[MBL]plasma and genotype (Kruskal-Wallis $p<0.0001$).
The two dashed lines represent MBL concentrations of 1500 ng/ml and 80 ng/ml respectively. Almost all individuals with wild type haplotype demonstrated MBL levels higher than 1500 ng/ml, while almost all heterozygous individuals have MBL levels above 80 ng/ml.

Due to the insignificant impact on MBL level of the L/H promoter variant and the more pronounced influence of the Y/X polymorphism, for simplicity, haplotypes were divided into either YA/YA, YA/XA or XA/XA for the wild type individuals, YA/YO or XA/YO for heterozygous individuals and YO/YO for individuals homozygous for exon 1 mutation. The effect of the individual promoter

polymorphism –221 X/Y on MBL levels was examined and these data are presented in Figure 4-6. The medians of the MBL concentrations in YA/YA, XA/YA and XA/XA individuals were 4197, 3653 and 2589 ng/ml respectively. The presence of the XA variant on one strand in combination with an exon 1 mutation on the other, significantly reduced MBL plasma level. The Median MBL levels in XA/YO individuals (289 ng/ml) were significantly lower than YA/YO (929 ng/ml). The median MBL concentration for 35 YO/YO individuals was 36 ng/ml, which was significantly lower than in all other groups.

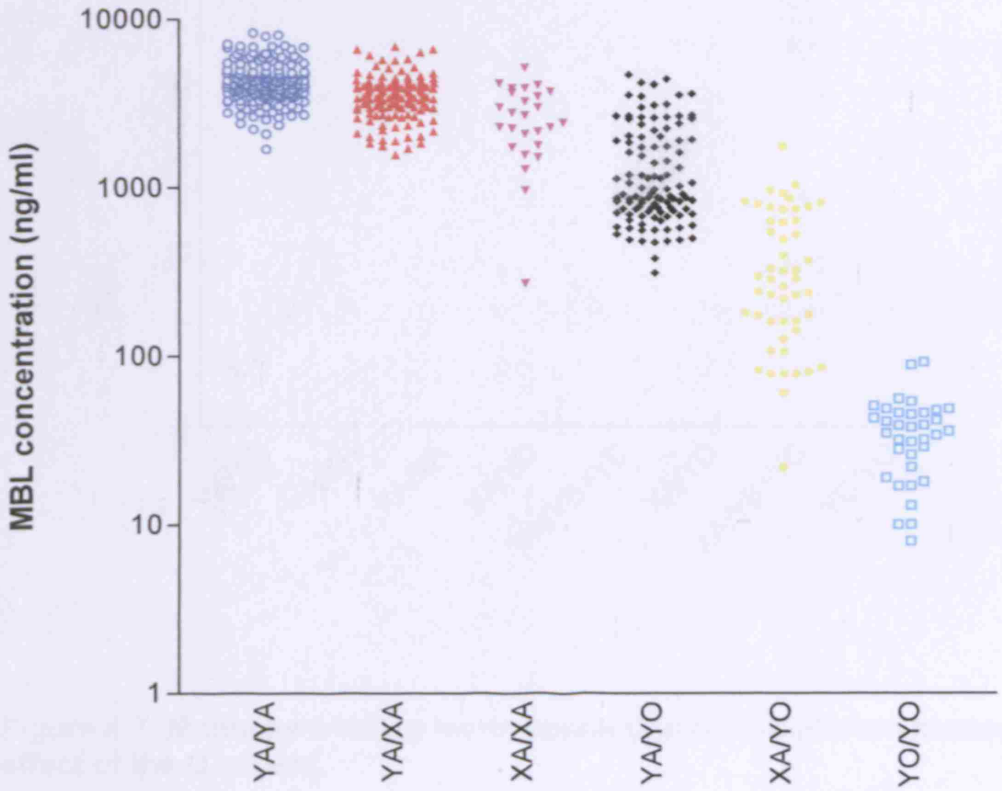


Figure 4-6. Mannose-binding lectin levels in plasma plotted in relation to MBL2 X/Y and A/O variants.

Log_{10}[MBL] plasma is displayed against MBL haplotype (Exon 1 and X/Y promoter polymorphisms). Individual values are shown.

Due to higher MBL levels in individuals with the D mutation, we plotted the D mutation and C+B mutations separately. The D containing haplotypes always had higher levels than the B and C containing haplotypes. This is shown in Figure 4-7 and suggests that for population studies it is possible to consider three haplotype grouping corresponding to three phenotypic categories. Thus YA/YA, YA/XA,

XA/XA, and YA/YD individuals are MBL "sufficient" individuals; YA/YB+YC and XA/YD individuals have intermediate MBL levels and XA/YB+YC and YO/YO individuals are MBL deficient.

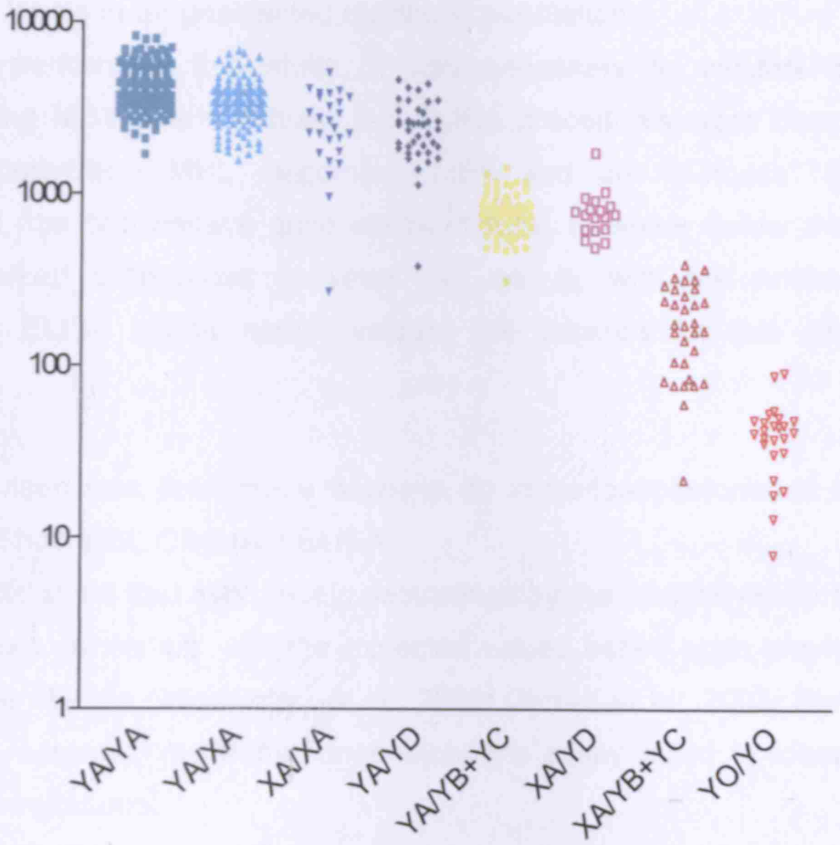


Figure 4-7. Mannose-binding lectin concentrations in plasma plotted to show effect of the D variant.

4.4 Discussion

This study was designed to analyse the relationship between the MBL2 genotype and MBL levels in an unselected childhood population.

Prior to performing this study, it was necessary to validate a method for determining MBL levels. Initially two ELISA procedures were compared, namely the AntibodyShop MBL Oligomer ELISA and an "in-house" ELISA. Above 800ng/ml, the two assays gave similar results. However below this value, there were marked differences between the assays, with the AntibodyShop MBL Oligomer ELISA giving higher values. The reasons for this discrepancy are unclear.

A comparison was then made between an immunonephelometric assay and the AntibodyShop MBL Oligomer ELISA.

Our results show that MBL levels determined by the AntibodyShop Oligomer MBL ELISA were consistent with the expected values based upon previous genotype-phenotype studies (Minchinton *et al.*, 2000; Garred *et al.*, 2003; Steffensen *et al.*, 2000). In contrast, the immunonephelometric assay failed to identify individuals with MBL mutations.

The MBL ELISA uses a single monoclonal antibody 131-01 for both sides of the sandwich. This antibody recognises MBL epitopes present in the multimeric conformations of the molecule. A study demonstrated that by changing clone 131-01 as capture antibody in the MBL ELISA detection system (Garred *et al.*, 2003) with another monoclonal antibody clone 131-11 (State Serum Institute) and still using the clone 131-01 as detector antibody it was possible to detect MBL antigen from structural homozygous individuals in amounts observed from A/O heterozygotes. Normal A/A MBL sera with different promoters contained predominantly higher order oligomers, while A/B, A/C and A/D sera contained differing degrees of higher or lower oligomers depending on the promoter type on the normal A haplotype. Sera containing homozygous or compound heterozygous variant MBL appeared to contain only lower order oligomers (Garred *et al.*, 2003). The poor agreement between two different assays suggests that the immunonephelometry assay is capable of detecting lower order MBL multimers or

even MBL monomers. Being an epitope-targeted assay, it may provide a direct indication of total MBL protein present in a plasma sample. As a result of this initial work, the AntibodyShop Oligomer MBL ELISA has been employed for subsequent studies. Since this study was completed AntibodyShop have decided to market two MBL ELISA kits. The first kit is the one described in this study, which is called the MBL Oligomer ELISA kit. The second assay is called the MBL mannan-binding ELISA kit in which mannan is used instead of anti-MBL antibody. The MBL mannan-binding ELISA kit is measuring MBL which is functionally active.

MBL levels determined in our study of children are comparable with those reported for other normal adult populations. Levels of circulating MBL, as assessed using different assays have been reported for Japanese, Caucasoids, African, Australian and Eskimo populations. The levels of MBL in Caucasians using Functional Mannan-binding assays have revealed mean MBL levels of 4.48 μg/ml (range 0-15.73) (Aittoniemi *et al.*, 1999) and 3.97 μg/ml (Aittoniemi *et al.*, 1997). Using an ELISA, Caucasoids had a median MBL of 0.99 μg/ml (range 0-4.89) (Madsen *et al.*, 1994). Using an ELISA but with different antibodies in a survey of 1085 Japanese gave a mean MBL level of 1.72 μg/ml (range 0.07-6.40) (Terai *et al.*, 1993). Eskimos had median MBL levels of 2.98 μg/ml (range 0-8.24) (Madsen *et al.*, 1994; Garred *et al.*, 1992), and Africans had a median MBL of 0.58 μg/ml (range 0-13.92)(Garred *et al.*, 1992). Another normal African population tested using the double-antibody assay gave a range of 0-4.05 μg/ml (Madsen *et al.*, 1998). In a UK population, a time-resolved immunofluorometric assay (TRIFMA) revealed mean MBL levels of 1.22 μg/ml (Crosdale *et al.*, 2000), and with the same assay, the MBL range in a Danish Caucasoid population was 0.002-5.48 μg/ml (Steffensen *et al.*, 2000). In a study of 189 indigenous Australians the median MBL level was 3.06 μg/ml reflecting the virtual absence of exon 1 mutations in this population (Turner *et al.*, 2000). These studies indicate that MBL levels are influenced by both the assay system employed and the ethnic composition of the populations studied.

As expected, both coding and promoter MBL2 variants were shown to be significantly associated with MBL levels. These results confirm previous reports

that A/A, A/O and O/O coding genotypes are associated with high, intermediate and profoundly reduced levels of MBL, respectively (Madsen *et al.*, 1995). Similarly, significant associations between the type of coding mutation and MBL levels were also observed. A/D individuals had significantly higher MBL levels than A/B or A/C individuals with some having levels comparable to A/A individuals as previously described (Minchinton *et al.*, 2002). At the molecular level, in contrast to the B and C mutations, the D mutation does not involve the substitution of the Glycine residue of a Gly-X-Y repeat in the MBL sequence. However, there is a difference in size between the wild type Arg and the substituted amino acid Cys. It has been proposed that this may affect the stability of the molecule, and that this may lead to accelerated degradation (Madsen *et al.*, 1994). Our observation shows that some A/D individuals have virtually normal MBL levels. Individuals carrying the C allele were found to have the lowest MBL levels amongst heterozygotes, but their numbers were too low to allow for statistical confirmation of this trend.

In keeping with the previous reports, we also observed a broad range of MBL levels for the A/A and A/O genotypes. Following the stratification of wild-type individuals according to promoter haplotype, it was observed that only the LXA haplotype was associated with a significant reduction in MBL levels. There was no significant difference in MBL levels between the wild type individuals carrying the LYA haplotype compared with the HYA haplotype. The effect of promoter haplotype on MBL levels was more evident in coding mutation heterozygotes than in wild type individuals. Our findings suggest that the LXA variant, but not LYA, significantly influences MBL levels. As the LYA haplotype encompasses both the LYPA and LYQA haplotypes, it is possible that stratification of LYA individuals according to LYPA and LYQA might yield further associations with variation in MBL levels. This was not the case in this study and supports the findings of Madsen *et al.* who failed to show an effect of the +4 P/Q polymorphism on MBL levels (Madsen *et al.*,1995). LYPA homozygosity is uncommon in Caucasoids, and the differential effects of LYPA and LYQA have only been studied in a Mozambique population due to the higher frequency of both haplotypes (Madsen *et al.*, 1998).
It was also shown that 26 A/A individuals with the LXPA/LXPA promoter had a mean MBL level of 2589 ng/ml. This was lower than in other wildtype haplotypes

but not as low as those seen in individuals heterozygous for MBL2 structural gene mutations (Madsen *et al.*, 1995; Steffensen *et al.*, 2000; Minchinton *et al.*, 2002).

In summary, these results have highlighted the influence of assay type in phenotypic analyses of MBL. The results presented in this chapter also confirm and extend previous reports of the influence of MBL 2 genetic variants on MBL levels. The nature of coding mutation influences levels of circulating MBL. We confirm that there is significant variability in MBL levels, some of which is explained by the MBL2 promoter variants. There appeared little difference between the HYA and LYA variants, whereas the LXA variant is associated with reduced MBL levels. This variation is most apparent with the alleles corresponding to the –221 X/Y polymorphism rather than those associated with the –550 H/L polymorphism. In light of the variability in MBL levels in individuals with identical coding genotype and the influence of promoter variants on MBL levels, it is clear that any future studies of MBL2 genotype, MBL levels and disease must evaluate both promoter and coding polymorphisms.

This study will provide the largest reference dataset for MBL genotype/phenotype relationships in an unselected UK population. As such it permits the meaningful selection of cut offs for low, intermediate and high-normal MBL levels in plasma.

Chapter 5 MBL in Clinical studies: clues to its possible role as an inflammatory modulator

In the literature MBL has been studied in a great diversity of diseases. In this chapter MBL deficiency and it's role in determining the susceptibility to and severity of meningococcal disease, sepsis and cystic fibrosis has been addressed.

5.1 MBL and meningococcal disease

5.1.1 Introduction

Neisseria meningitidis is the most common cause of bacterial meningitis in countries offering vaccination against *Haemophilus influenzae* type B. It is an exclusively human pathogen spread by close contact and is commonly present in the nasopharynx of up to 20% of the human population (Cartwright, 1991). In susceptible individuals, meningococci migrate from the nasopharynx to the vascular system, where they employ a number of mechanisms to avoid destruction by the immune system. Organisms may migrate to the central nervous system causing inflammation of the meninges (meningitis) or to synovial joints. In the absence of sepsis, the mortality associated with treated meningitis is low (<5%)(Rosenstein *et al.*, 1999). Sepsis caused by the meningococcus is, however, associated with a much higher mortality which may be as high as 20% (Rosenstein *et al.*, 1999). These patients present with evidence of inflammation, disseminated intravascular coagulation and hypovolaemia due to injury to the vascular endothelium (Deuren *et al.*, 2000) and shock.

Antibodies against *N.meningitidis* appear to be protective. This is supported by the finding that children younger than 1 year in whom the major burden of meningococcal disease occurs have a low prevalence of antibodies directed against *N.meningitidis* (Cartwright, 1997). In spite of this, the incidence of meningococcal disease remains low (Rosenstein *et al.*, 1999).

The protection afforded to humans by antibodies does not appear to be through their interaction with phagocytes as patients with phagocytic deficiencies or defects are not especially susceptible to infections caused by *N. meningitidis*. It would seem that it is the complement system which is particularly important in protection against this organism. Deficiencies of terminal complement components (C6 to C9) results in increased susceptibility to *N. meningitidis* and such patients are said to have a 50 fold increased risk of infection (Figueroa *et al.*, 1993). However these patients may also have less severe infections (Figueroa *et al.*, 1993). Individuals who are deficient in the alternative pathway protein properdin present later in life

with meningococcal disease and appear less likely to survive the disease although some patients with recurrent disease have been reported (Fijen *et al.*, 1999).
The rarity of complement deficiencies suggests that they are unlikely to contribute significantly to the overall incidence of meningococcal disease.
In contrast MBL deficiency, which occurs at high frequency, could contribute to the incidence of *N.meningitidis* disease since the protein activates complement and in individuals without exon 1 mutation is present at adult levels in the first year of life.
MBL levels were measured in a retrospective study of Norwegian army recruits and young adults (Garred *et al.*, 1993) who had received an experimental vaccine against one serogroup of meningococcus, but had gone on to develop the disease. Convalescent seram from 99 survivors was compared to a control group of 49 healthy blood donor controls. There was no significant difference between the levels of MBL in patients and controls. There was also no increase in the frequency of infection in individuals with very low levels of MBL (<0.1 μg/ml and therefore likely to be homozygous for variant alleles). It was concluded that MBL was not associated with susceptibility to meningococcal disease.
A second population study conducted in the UK (Hibberd *et al.*, 1999) analysed MBL genotypes in a prospective hospital population of 194 patients and a retrospective population of 72 survivors of meningococcal disease. These were compared to 272 patients with non-infectious illnesses and 110 healthy controls. It was found that the overall frequency of individuals homozygous for MBL variant alleles in these populations was increased from 1.5 in controls to 7.7% in the patients with an odds ratio of 6.5 (2.0-27.2, 95% confidence interval). In the community study the frequency of homozygous variants was 8.3% compared to 2.7% in controls with an odds ratio of 4.5 (0.9-29.1, 95% confidence interval). It was concluded that MBL variant alleles might be implicated in up to 32% of meningococcal cases. There was apparently a trend towards less severe disease in homozygous carriers of MBL mutant alleles. Some support for these observations comes from the study of an extensive family pedigree in which MBL homozygosity was associated with susceptibility to meningococcal infection (Bax *et al.*, 1999). However, these observations are still controversial since, as pointed out by Tang and Kwiatowski (1999), the codon 52 and 54 mutation frequencies were rather low in the non-infectious admission control group used by Hibberd *et al*

(1999). Further epidemiological studies are required to define the role of MBL in meningococcal disease.

The purpose of the study described in this section was to investigate the relationship between MBL2 exon 1 and promoter -221 polymorphisms and the susceptibility to meningococcal disease.

Acknowledgment

All experimental work on DNA amplification was done by Dr. D.L.Jack (Division of Genomic Medicine, Sheffield).

All clinical data were provided by Dr. R.Read (University of Sheffield Medical School, UK).

5.1.2 Materials and Methods

5.1.2.1 Samples

The Meningococcal Reference Unit (MRU) for England and Wales offers a service for polymerase chain reaction (PCR) detection of *Neisseria meningitidis* in blood and cerebrospinal fluid (CSF) samples from patients with suspected meningococcal disease. From July 1998 to November 1999, all whole blood samples were archived from patients who were confirmed to have meningococcal disease, either by culture or by PCR detection of *N. meningitidis* in the blood or CSF. The Ethics Committees of the Public Health Laboratory Service for England and Wales and of the South Sheffield Health District approved the final protocol of the study of the meningococcal patients. The serogroup of the infecting organism, whether the patient died or survived and the age of the patient were recorded. After clinical information had been collated, samples were coded so that patients could no longer be identified.

The control population (ALSPAC study) had been described in Chapter 4.

5.1.2.2 MBL genotyping

MBL genotypes were determined by heteroduplex analysis as described previously in Chapter 2.7 (Jack *et al.*, 1997; Turner *et al.*, 2000) in 770 meningococcal disease patients and 1046 individuals in the control cohort. For the purposes of grouping, individuals with the C mutation were combined with patients carrying the B mutation, since these mutations are quantitatively and qualitatively similar (Garred *et al.*, 1999). In the analysis of the risk of death from meningococcal disease, we grouped together all three structural mutations, defined as 'O', to preserve high enough case numbers for analysis. Genotype frequencies for controls were taken from the ALSPAC study described in Chapter 4.

5.1.2. 3 Statistical Analysis

The chi-square test was used to analyse the effect of genotype on susceptibility to meningococcal disease and the risk of death after stratification by MBL genotype.

Logistic regression analysis was used to determine the effect of MBL structural genotype, IL1 genotypes, age, infecting serogroup, interactions within IL1 genotypes and interactions between MBL and IL1 genotypes.

Stata 7 software (Stata Corp., College Station, Texas) was used for statistical analyses.

5.1.3 Results

5.1.3.1 Susceptibility to meningococcal disease

We compared the frequency of MBL genotypes between controls and patients with meningococcal disease Figure 5-1 and Table 5-1.

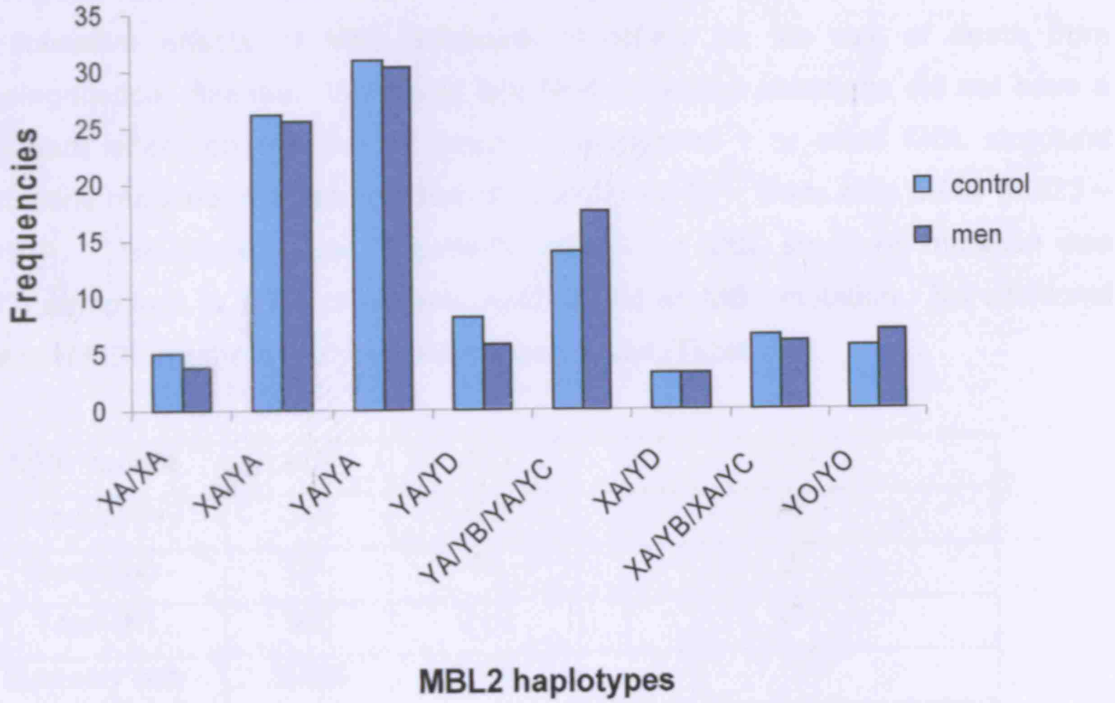


Figure 5-1. The distribution of short MBL haplotypes in a meningococcal disease cohort and a general population control group.

Turquoise bars represent ALSPAC samples, blue bars represent meningococcal disease cohort.

The observed frequency of MBL homozygous structural mutations was 7.0 % in patients compared to 5.6 % in controls. The frequency of individuals heterozygous for exon 1 variants of MBL was 33.6 % compared to 32.2 % in controls. When the analysis included promoter polymorphisms no significant differences in MBL allele

distribution between patients and controls was found (p = 0.154, χ^2 test). Patients were also stratified by age and compared to controls. An apparent difference in allele distribution between patients and controls for the individuals between 10 and 19 years (p = 0.017) was observed. However, odds ratio analysis found no significant change in the risk of meningococcal disease for any given MBL genotype in this age group.

5.1.3.2 Likelihood of death from meningococcal disease

A logistic analysis was used to investigate the dominant, recessive and multiplicative effects of MBL structural mutations on the risk of death from meningococcal disease. We found that MBL structural mutations did not have a dominant effect on the risk of death. Carriage of 1 or more MBL structural mutations resulted in a reduced risk of death (p <0.001, Odds ratio 0.089 [0.023 – 0.352]). The mortality rate in patients without an MBL structural mutation was 9.0% compared to 7.1% of patients who carried an MBL mutation. No additional effect of MBL promoter polymorphisms was found (Table 5-2).

MBL variant	A/A	A/O	O/O
Survival (N)	348	196	37
Dead (N)	37	14	3
Total (N)	385	210	40
Mortality rate	9.0%	6.7%	7.5%

Table 5-2. Likelihood of death from meningococcal disease

5.1.4. Discussion

Low levels of complement-activating immunoglobulin or deficiency of the terminal components of the complement pathway appear to increase the susceptibility of humans to meningococcal disease (Figueroa, Andreoni, Densen, 1993). It therefore appeared plausible that a genetic deficiency of another complement activator, MBL, might similarly increase susceptibility to meningococcal disease. Reports of such an association are inconclusive, although in a reasonably large study an association has been reported (Hibberd *et al.*, 1999). In the present study, no association was found between MBL genotype and an altered risk of acquiring meningococcal infection.

The major difference between our study and that of Hibberd *et al.* (1999) appears to be the composition of the control group. The frequency of homozygous MBL structural gene mutations in meningococcal patients was 7.0%. This is similar to both frequencies reported by Hibberd *et al.* (7.7% and 8.3%). Therefore the differences in outcome between the studies in terms of disease susceptibility must be due to differences in the control group. In the study by Hibberd *et al.* (1999) two control groups were used; the first were patients admitted for non-infectious reasons and the second were unrelated neighbours or friends of the index case (1.5% - 2.2% frequency of structural homozygotes, respectively). Our control population was derived from the unselected general population ALSPAC cohort and revealed a much higher frequency of homozygous variant MBL alleles (5.6%). The importance of the frequency of MBL haplotypes within the control group is highlighted by our observation that if the control group described by Hibberd *et al.* (1999) was used in comparison to our disease cohort, an OR for susceptibility to infection would have been of 1.94 (1.4 – 2.69) for MBL structural heterozygotes and 6.08 (2.17 – 17.03) for homozygotes.

The control group used in our study represents an 'at risk' group rather than a 'disease-free' group. As such this may underestimate the effect of MBL mutations on meningococcal disease susceptibility. MBL deficiency could be conferring a small increased risk for meningococcal disease. However, any increased risk is of

a different order of magnitude to that observed for terminal complement deficiencies and may not be as specific for *N. meningitidis*.

We found that MBL structural polymorphisms reduced the risk of death from meningococcal disease in IL-1 deficient individuals after correction for other factors known to influence survival (Read *et al.*, 2003). The effect we observed was consistent with genetic dominance of MBL structural mutations. A similar effect for homozygotes, but not heterozygotes, which did not achieve statistical significance, was observed previously (Hibberd *et al.*, 1999). Our data suggest that individuals with MBL variant alleles, whether heterozygous or homozygous, would be protected from death as a result of infection.

The mechanism through which MBL might operate to modify survival is unclear. It is believed that complement deficiencies increase susceptibility to meningococcal disease, but reduce severity by limiting either LPS release or the production of inflammatory mediators such as C5a or C3a (Figueroa *et al.*, 1993). Whilst MBL can recognise meningococci and activate the complement system (Jack *et al.*, 1998; Jack *et al.*, 2001), the absence of a major effect of MBL deficiency on meningococcal susceptibility might suggest that this is not the principle explanation for the increased survival of patients.

Activation of the inflammatory response is important in the pathogenesis of meningococcal disease. The release of higher levels of inflammatory cytokines has been noted in more severe meningococcal disease and polymorphisms in the genes that encode or control the expression of TNFα (Estabrook *et al.*, 2004) and IL1β (Read *et al.*, 2000; Read *et al.*, 2003) have been reported to modify disease susceptibility or severity. MBL can target *N. meningitidis* to phagocytes and modulate the inflammatory response of these cells *in vitro* (Jack *et al.*, 2001; Sprong *et al.*, 2004). The influence of IL1 polymorphisms on the survival of patients (Read *et al.*, 2003; Read *et al.*, 2000) suggests that the cellular inflammatory response to meningococcal infection is critical in determining disease severity. Polymorphisms in the IL1 system that promote effective early release of IL1β and a favourable molar ratio of IL1 receptor antagonist are associated with an increased chance of survival. Concentrations of MBL within the normal

physiological range lead to increased IL1 release from monocytic cells, whereas levels of MBL expected during an acute phase response lead to reduced IL1 responses. IL1β modulation by MBL could be deleterious in meningococcal disease because IL1 may be downregulated inappropriately by high levels of MBL or could be modulated at an unfavourable molar ratio to IL1 receptor antagonist.

In conclusion, MBL genotype does not appear to be a major factor in determining susceptibility to meningococcal disease although age may be a factor. Reduction of MBL levels through structural gene polymorphisms may be a mechanism for protecting individuals against inflammation-mediated damage in septic conditions.

5.2 Mannose-binding lectin polymorphisms in severe sepsis

5.2.1 Introduction

Systemic inflammatory response syndrome (SIRS) is associated with different overlapping scenarios, comprising invasive infection, dissemination of microbes secondary to injury, shock, and activation of inflammation by apparently non-infectious events (Rangel-Frausto *et al.*, 1995). SIRS can be self-limiting or can, in infected patients, progress to severe sepsis and septic shock (Bone *et al.*, 1997). Sepsis is the most common cause of death in adult intensive care units (ICU). Mortality rates remain high, death often being attributable to a dysfunctional innate immune response with recurrent episodes of sepsis and/or excessive systemic inflammation (Hotchkiss *et al.*, 2003).

It is now well recognised that individuals differ considerably in their ability to resist and respond appropriately to infection and that inherited factors are important determinants of such differences (Sorensen *et al.*, 1988). Recent advances in molecular biology have encouraged efforts to elucidate the role of genetic variation in the pathogenesis of sepsis. Much of this work has focused on the effect of various cytokine polymorphisms on the incidence of, and outcome from sepsis although such studies have often produced conflicting results (Gordon *et al.*, 2004; Mira *et al.*, 1999; Stuber *et al.*, 1996). Recently the presence of MBL2 variant alleles has been associated with the development of sepsis in adult patients admitted to intensive care (Garred *et al.*, 2003) and in children (Fidler *et al.*, 2004) with the systemic inflammatory response syndrome (SIRS).

In the study by Garred *et al.* (2003) of 272 patients with SIRS, different MBL genotypes were compared with respect to microbiology, sepsis development, and survival. The study revealed that a highly significant proportion of the patients with sepsis carried MBL variant alleles, compared with the patients without sepsis. Further analysis showed that the patients carrying MBL variant alleles also had a high risk of developing severe sepsis and septic shock. A gradual decrease of serum MBL was seen with increased severity of sepsis (SIRS, sepsis, and septic shock). A significantly increased proportion of the patients carrying MBL variant

alleles were culture positive in a gene dose-dependent manner. MBL deficiency was associated with both gram-negative and gram-positive bacteria. Patients with the highest levels of MBL (YA/YA) were the most protected against fatal outcome. The increased risk of mortality in patients carrying MBL variant alleles was present in both the sepsis group and the non-sepsis group.

The study by Fidler *et al.* (2004) was of development and severity of SIRS in 100 children admitted to intensive care. 42 patients had variant MBL alleles and were significantly over-represented amongst the 59 cases that developed SIRS. For patients with infections, variant MBL alleles were associated with increased systemic response (infection, sepsis and septic shock).

The purpose of the study described here was to investigate the relationship between MBL2 exon 1 and promoter -221 polymorphisms, together with plasma levels of the encoded protein and outcome in adults with severe sepsis and septic shock.

Acknowledgment
All clinical data as well as albumin levels were provided by Dr. A. Gordon (William Harvey Research Institute, Barts, UK).

5.2.2 Materials and Methods

5.2.2.1 Samples

Patients were recruited from a total of eight different ICUs in the South of England. The study was approved by the local ethics committees and written consent from patients, or written assent from the next of kin, was obtained as appropriate.

Adult Caucasian patients (18-80 years) with severe sepsis or septic shock, as defined by the American College of Chest Physicians/ Society of Critical Care Medicine (ACCP/SCCM) Consensus Conference 1992, were recruited. Patients who were immunosupressed prior to developing sepsis were excluded, including those with known HIV infection, haematological malignancy, neutropenia (white cell count <1 $x10^9$/l) or chronic liver failure (according to APACHE II definition[22]) and patients who had received chemotherapy, immunosuppressants or systemic steroids in the previous six months.

5.2.2.2 Clinical information

Clinical information recorded for each patient included demographic details (age, sex), details of acute illness, and APACHE II score[22] on admission to ICU.

5.2.2.3 Genotyping of samples for MBL polymorphisms

Whole blood was collected in ethylene diamine tetraacetic acid (EDTA) tubes from all patients enrolled in the study for DNA extraction, using QIAamp™ DNA extraction kits (Qiagen UK Ltd, Crawley, Sussex).

MBL exon 1 and promoter polymorphisms were determined by heteroduplexing procedures as previously described in Chapter 2.7. In the analysis we grouped together all three structural mutations, defined as 'O', to preserve high enough case numbers for analysis

5.2.2.4 Measurement of MBL levels

In three ICUs blood was also collected in EDTA on the day of recruitment and one week later if the patient was still on ICU, for measurement of plasma levels of MBL. Plasma was separated immediately and frozen at –20°C until analysis using the MBL Oligomeric ELISA kit by AntibodyShop as described in Chapter 2. 5.

Genotype frequencies for controls were taken from two previously published studies: 353 Caucasian adult blood donors (age range 23-62 years) from Oxfordshire (Roy *et al.*, 2002) and 302 newborns from Avon, UK (Mead *et al.*, 1997).

5.2.2.5 Statistic analysis

Genotype data were analysed using the chi-squared test. Continuous data were not normally distributed and are therefore expressed as medians with ranges and analysed using Mann-Whitney U or Kruskal-Wallis tests and Spearman rank correlation, as appropriate. Multiple logistic regression analysis was used to investigate the association of mortality and genotype after adjusting for age and reason for ICU admission (medical / surgical). Results were considered statistically significant if the p value was less than 0.05. SPSS (version 11) for Windows and GraphPad Instat V02.04 were used for analysis.

5.2.3 Results

5.2.3.1 Patient demographics

174 adult Caucasian patients with severe sepsis or septic shock were recruited (providing 80% power to detect a relative risk for mortality of 2.5 at $p<0.05$). The median age was 63.5 years (19-80), 110 (63.2%) were male and the median APACHE II score was 18 (4-44). Fifty patients (28.7%) died on the ICU.

5.2.3.2 MBL haplotypes and serum levels

The exon 1 polymorphisms (A/O or O/O) were significantly more common in the patients with severe sepsis and septic shock than in normal healthy adults recruited from the Oxford region (54.6% v 39.7%, $p=0.001$) as well as when promoter allele frequencies were also compared between control adults and septic patients ($\chi^2 = 28.5$, df 5, $p<0.0001$) (Figure 5-2).

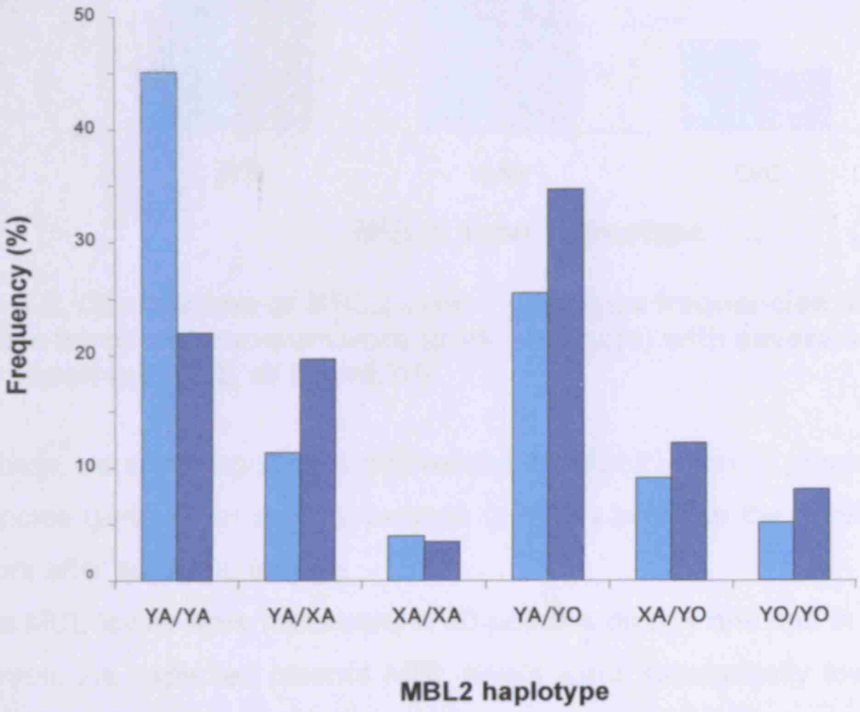


Figure 5-2. Comparison of MBL2 haplotype frequency in an adult UK control group (sky blue bars) and patients with severe sepsis and septic shock (dark blue bars) ($\chi 2 = 28.5$, df 5, $p<0.0001$).

However there was no significant difference in MBL2 haplotype frequency (χ^2 =3.2, df 5, p=0.67) or exon 1 polymorphism frequency (χ^2 = 0.6, df 2, p=0.74) between survivors and non-survivors (Figure 5-3).

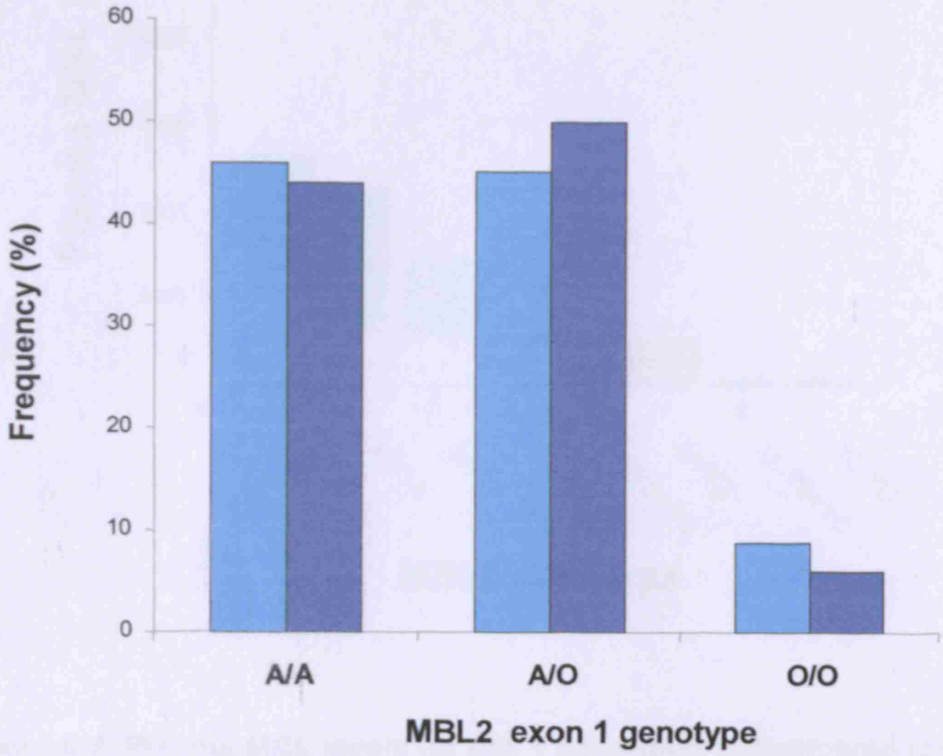


Figure 5-3. Comparison of MBL2 exon 1 genotype frequencies in survivors (sky blue bars) and non-survivors (dark blue bars) with severe sepsis and septic shock (χ2 = 0.6, df 2, p=0.74)

Also there were no significant differences in MBL2 exon 1 promoter haplotype frequencies (p=0.49) or exon 1 variants (p=0.88) between the survivors and non-survivors after adjusting for age.

Plasma MBL levels were measured in 80 patients on day one and in 30 patients on day seven. As expected plasma MBL levels were substantially lower in patients with the MBL2 structural gene mutations (either heterozygotes (A/O) or homozygotes (O/O) than in those homozygous for the wild-type (A/A) (day 1, 140 v 1659μg/l, p<0.001 and day 7, 164 v 2079.2μg/l, p<0.001). There was a strong relationship between MBL2 haplotype and plasma MBL concentration (p<0.001) (Figure 5-4).

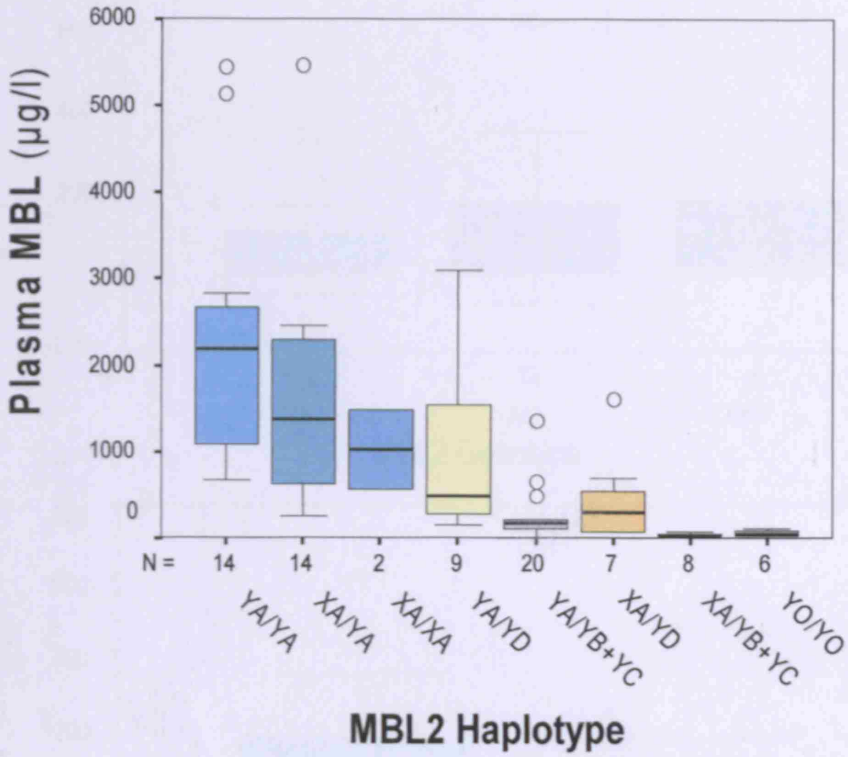


Figure 5-4. Plasma MBL levels on day 1 according to haplotype (p<0.001).
(Line=median, box= interquartile range, O=outliers. All data included in analysis.)

Plasma MBL levels were an average of 86.9% (95%C.I. 32.4 – 141.3%) higher on day seven compared to day one. The magnitude of the increase in MBL levels over time did not appear to be influenced by genotype or by the resolution of severe sepsis / septic shock (Figure 5-5).

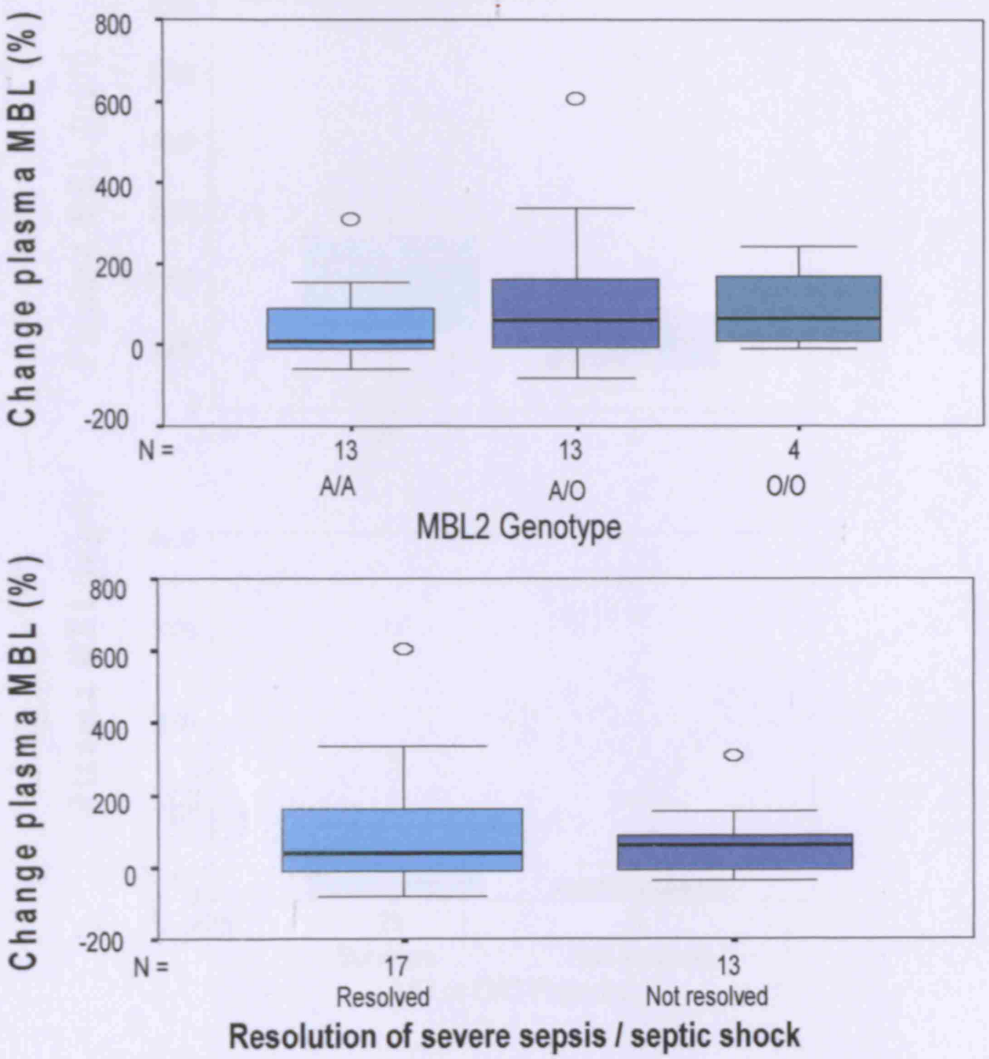


Figure 5-5. Percentage change in plasma MBL levels from day one to day seven according to MBL2 exon 1 genotype (above) and resolution of severe sepsis and septic shock (below).

In the group as a whole there was a higher mortality rate in those with MBL levels <1000µg/l than in those patients with levels >1000 µg/l (47.2 v 22.2%, p=0.05).

In A/A homozygous patients, MBL levels were significantly higher in survivors compared to non-survivors (2188 v 1042µg/L, p=0.035) whereas in those with a mutation the difference between survivors and non-survivors was not statistically significant (A/O or O/O) (179 v 111µg/l, p=0.54) (Figure 5-6).

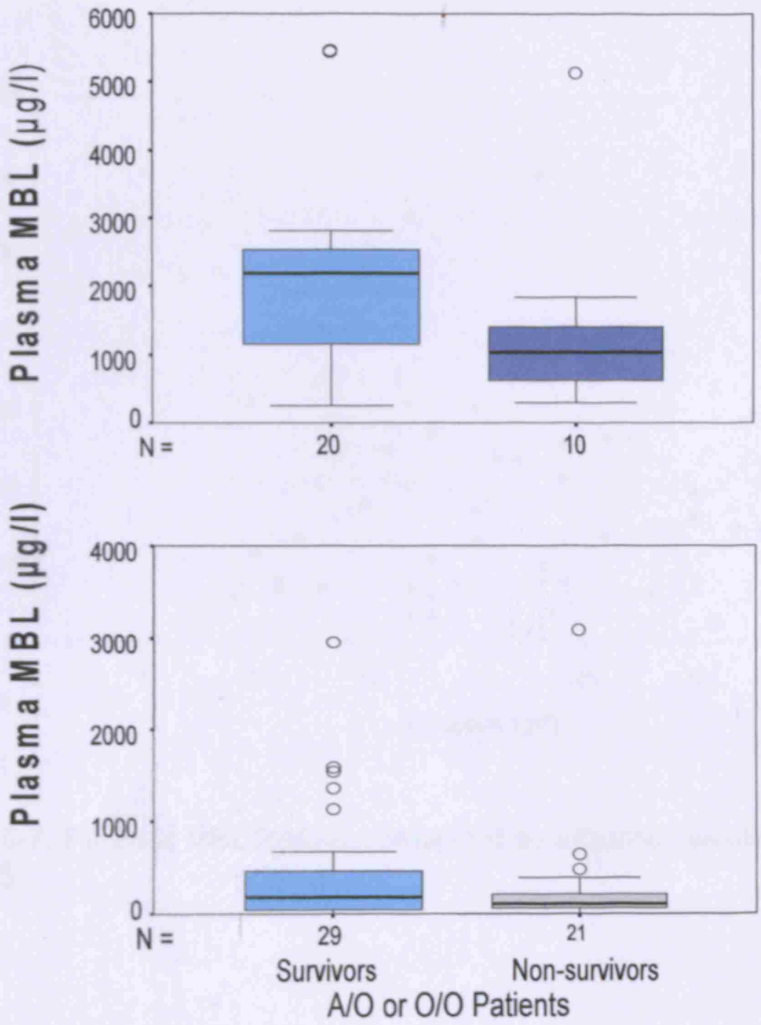


Figure 5-6. Day one plasma MBL concentrations in survivors and non-survivors (A/A patients above, p=0.035; A/O and O/O patients below, p=0.54)

Analysis of promoter variants revealed a trend towards increasing mortality in individuals heterozygous or homozygous for the X polymorphism in patients with an A/A genotype; YA/YA 21% (8/38), XA/YA 32% (11/34) and XA/XA 50% (3/6) mortality, although this was not statistically significant (p=0.21).

There was no correlation between MBL levels and age, APACHE II score or serum albumin level (Figure 5-7).

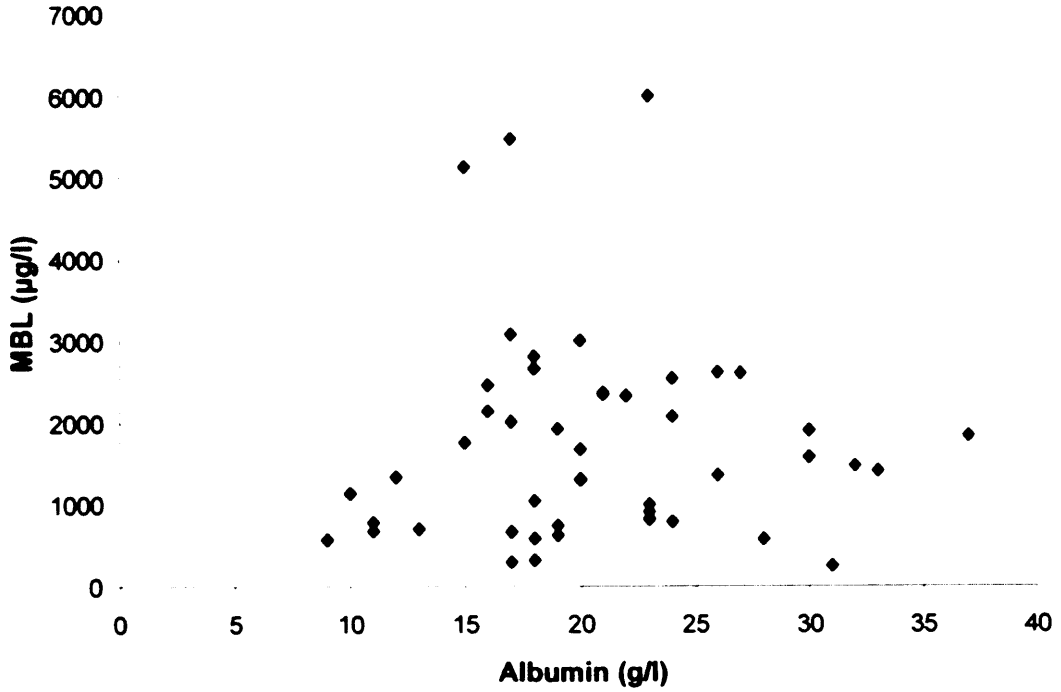


Figure 5-7. Plasma MBL levels compared to albumin levels in A/A patients (p=0.32)

5.2.4 Discussion

This study demonstrated that MBL2 variant alleles are significantly more common in adults with severe sepsis and septic shock than in two control populations. This observation is in keeping with the findings of two recent studies in which the variant MBL2 polymorphisms were associated with the development of sepsis, severe sepsis and septic shock in critically ill adults (Garred *et al.*, 2003) and in children with a systemic inflammatory response (Fidler *et al.*, 2004).

Despite this strong relationship between MBL2 genetic variants and susceptibility to severe sepsis/septic shock there was no demonstrable influence of MBL2 genotype on outcome. This is in contrast to a previous investigation in which the MBL2 variant alleles were associated with a worse outcome from critical illness (Garred *et al.*, 2003). However, these investigators recruited a mixed group of critically ill patients with SIRS, including a significant number who did not progress to severe sepsis/septic shock and more than a quarter who did not have a documented infection. In contrast in the study reported here, all patients were by definition already severely ill due to overwhelming infection. Moreover, in the study reported by Garred *et al.* (2003) the association between MBL deficiency and the development of infection was more significant than that between MBL deficiency and outcome. It seems possible, therefore, that the explanation for the worse outcome in patients with MBL2 variant alleles in that study may have been the more frequent acquisition of nosocomial infection and recurrent episodes of sepsis. The functionality of the MBL2 exon 1 and promoter polymorphisms has been well described (Steffensen *et al.*, 2000; Madsen *et al.*, 1995; Hansen *et al.*, 2003). This study has confirmed that these polymorphisms continue to influence circulating MBL levels even during the extreme physiological and metabolic derangement associated with severe sepsis and septic shock, although levels were lower than reported in other cohorts (Steffensen *et al.*, 2000; Ogden *et al.*, 2001). There was no correlation between MBL levels and age or illness severity as reflected by APACHE II score or albumin levels. Interestingly, only amongst A/A homozygote ("wild type") patients were MBL levels significantly lower in non- survivors than in survivors. This may in part be due to the influence of the X/Y polymorphisms as patients with an X allele tended to have a worse outcome.

In a previous study (Garred *et al.*, 2002) of long stay critically ill patients MBL levels rose significantly over time, with the largest increases being seen in survivors. In our study MBL levels also increased from day one to day seven, although due to the small number of day seven samples obtained from non-survivors it was not possible to determine whether this rise was greater in survivors than non-survivors. However, the increase was similar regardless of whether the severe sepsis or septic shock had resolved. The magnitude of the percentage increase in MBL levels did not appear to be influenced by genotype.

It is clear that during sepsis circulating MBL levels are subject to some alteration independent of genotype and that these variations may be influenced by the nature and severity of the disease process. Although knowledge of an individual's MBL2 genotype provides some indication of their susceptibility to infection and the likelihood of progression to severe sepsis, circulating levels of MBL protein seem to be more closely related to outcome in those with established severe sepsis, at least in A/A homozygotes.

5.3 MBL in cystic fibrosis

5.3.1 Introduction

Most of the morbidity and mortality in cystic fibrosis (CF) relates to lung disease (Koch and Hoiby, 1993), characterised by chronic infection with organisms such as *S. aureus*, *H. influenzae* and *P. aeruginosa* (Rajan and Saiman, 2002) and a sustained and deleterious inflammatory response. However, both onset and progression of disease are highly variable, even in subjects with identical *CFTR* mutations, leading investigators to search for modifier genes, such as alternative ion channels or proteins involved in host defence and inflammation. Mannose-binding lectin (MBL) has recently been identified as one such modifier (Garred *et al.*, 1999; Gabolde *et al.*, 1999). Together with the mucociliary escalator, professional phagocytes and other proteins (lysozyme, lactoferrin, defensins, complement), collectins form part of the first line of airway defence, before the adaptive immune systems come into play.

The first suggestion that MBL may modify CF lung disease came from a study of 149 patients by Garred *et al.* (1999). Although the frequency of MBL mutations was similar to that observed in the healthy population, lung function, measured by FEV_1 and FVC, was significantly lower in the group with MBL deficiency (both heterozygotes and homozygotes). A trend towards this was visible as early as 8 years of age, becoming significant by 16 years of age. Although *P. aeruginosa* has been identified as one of the major contributory factors in progression of lung damage in CF, in this study MBL- deficient patients did not have higher rates of pseudomonal infection. Rather, it appeared that when chronically infected with *P. aeruginosa*, MBL-deficient patients experienced a marked decline in lung function, which was not apparent in the MBL-sufficient group, and which appeared adversely to influence survival. The frequency of MBL mutations was significantly higher than would have been expected amongst 10 *B. cepacia* infected patients. A second study reported slightly different results. These investigators compared MBL-deficient CF patients homozygous for the ΔF508 mutation with controls closely matched for age (Gabolde *et al.*, 1999). In contrast to the earlier study, they found a significant reduction in lung function only in patients with MBL2 mutations. They

also reported a trend towards increased *P. aeruginosa* infection in these subjects but *B. cepacia* was not mentioned in this report.

These observations led to a search for possible mechanisms. Using bacteria obtained from the sputum of CF patients, Davies *et al.* (2000) were able to demonstrate that MBL did not bind significantly to the surface of either mucoid or non-mucoid *P. aeruginosa* (Davies *et al.*, 2000). In contrast, high levels of MBL binding to *B. cepacia* were observed. Activation of complement was demonstrated on the surface of the bacteria which could precede bacterial killing *in vivo.* This observation would support a role for MBL in the clearance of *B.cepacia*, and may explain the increased incidence of MBL-deficiency in patients infected with this organism in the Danish study of Garred and colleagues.

A third epidemiological study (Yarden *et al.*, 2004) found an association between MBL structural mutations and severity of cystic fibrosis lung disease in 112 Belgian and Czech patients. Individuals with AO or OO genotypes were more likely to have a severe pulmonary phenotype than wild type individuals. No association was found between the MBL genotype and the first age of infection with *P.aeruginosa*.

A patient with CF, severe bronchopulmonary *Pseudomonas aeruginosa* infection and MBL-deficiency, was treated with purified mannose-binding lectin intravenously (Garred *et al.*, 2002). The patient appeared to stabilise but did not improve dramatically. All the above studies focused on older children and adults. Based on our understanding of the functions of MBL, the protein may be important in the early stages of infection, before initiation of an adaptive immune response. This, together with the trend towards worse lung function in the children at the age of 8 years in the Garred study (2002) has led us to explore the effect of MBL deficiency in a UK paediatric CF population.

Acknowledgment

All clinical data and samples as well as the results for lung function tests were provided by Dr. J. Davies (Royal Brompton Hospital).

5.3.2 Materials and methods

5.3.2.1 Subjects

Children were recruited from the paediatric CF clinic at the Royal Brompton Hospital during 2000 and early 2001. At the time of a routine blood test, an extra 1-2 ml of blood was taken from all children in whom a sufficient sample could be obtained. The study was approved by the Ethics Committee of the Royal Brompton Hospital, Harefield and NHLI, and all parents or carers gave informed consent for participation.

5.3.2.2 Clinical data

Clinical data were obtained from the clinical databases, patients' hospital notes and computerised microbiology reports. Forced expiratory volume in the first one second (FEV_1) and forced vital capacity (FVC) were obtained from annual lung function laboratory records, corrected for sex, height and expressed as a percentage of predicted normal values (Rosenthal *et al.*, 1993). Lung function data were recorded for the following approximate time points: 6-7 years, 8-9 years, 10-11 years, 12-13 years and 14-16 years. Annual rates of decline were calculated on children with a minimum of 2 lung function recordings at least 1 year apart. In cases where data from more than 2 time points were available, the first and last recordings were used.

Microbiological data included the presence of positive cultures (sputum, cough swab or bronchoalveolar lavage) for *P. aeruginosa* (mucoid or non-mucoid and age at first isolation), *B. cepacia,* and *S. maltophilia*. *S. aureus* was not included due to its frequency and the fact that a majority of the children were on prophylactic anti-staphylococcal medication.

5.3.2.3 MBL Genotyping

Genotyping was performed separately for the exon 1 mutations and the promoter polymorphisms as described in the Chapter 2.7. In the analysis all three structural mutations were grouped together, and defined as 'O'.

5.3.2.4 MBL protein levels

MBL levels in serum were detected by a symmetrical sandwich AntibodyShop Oligomer ELISA Copenhagen, Denmark according to the manufacturer's instructions as described in Chapter 2. 5.

5.3.2.5 Statistical analysis

Data were analysed using SPSS v 10.0. Differences between groups were analysed with the Mann Whitney test, the Kruskal Wallis (KW) test or by Chi squared analysis. Fisher's exact test was carried out for groups containing less than 5 values.

5.3.3 Results

5.3.3.1 Patient demographics

No parent refused consent, although some children had insufficient blood available for some children and these individuals were not included. Samples from a total of 260 children (48% male) were available. MBL haplotypes were available on 249, serum MBL levels on 256 and both results in 245 cases. Median age at the time of enrolment was 8.5 [5.1;12.5] years. In 228 children, CFTR genotype data were available, as follows: Δ/Δ (58.8%), Δ/other (34.6%) and other/other (6.6%).

5.3.3.2 MBL haplotype distribution

The proportion of patients with no structural mutation (designated conventionally A/A) was 66.2%. Heterozygotes (A/O) made up 30.9%, and patients with 2 structural mutations (O/O) 2.9%. In patients with structural mutations, the B allele was found on 71% of chromosomes, with the other mutations occurring less frequently (C 11%, D 18%). The X promoter polymorphism was found in association with 28.2% of wild-type structural alleles. These frequencies and distributions are similar to those observed in other studies of both unselected subjects (ALSPAC) (Figure 5-8) and patients with CF (Garred *et al.*, 1999).

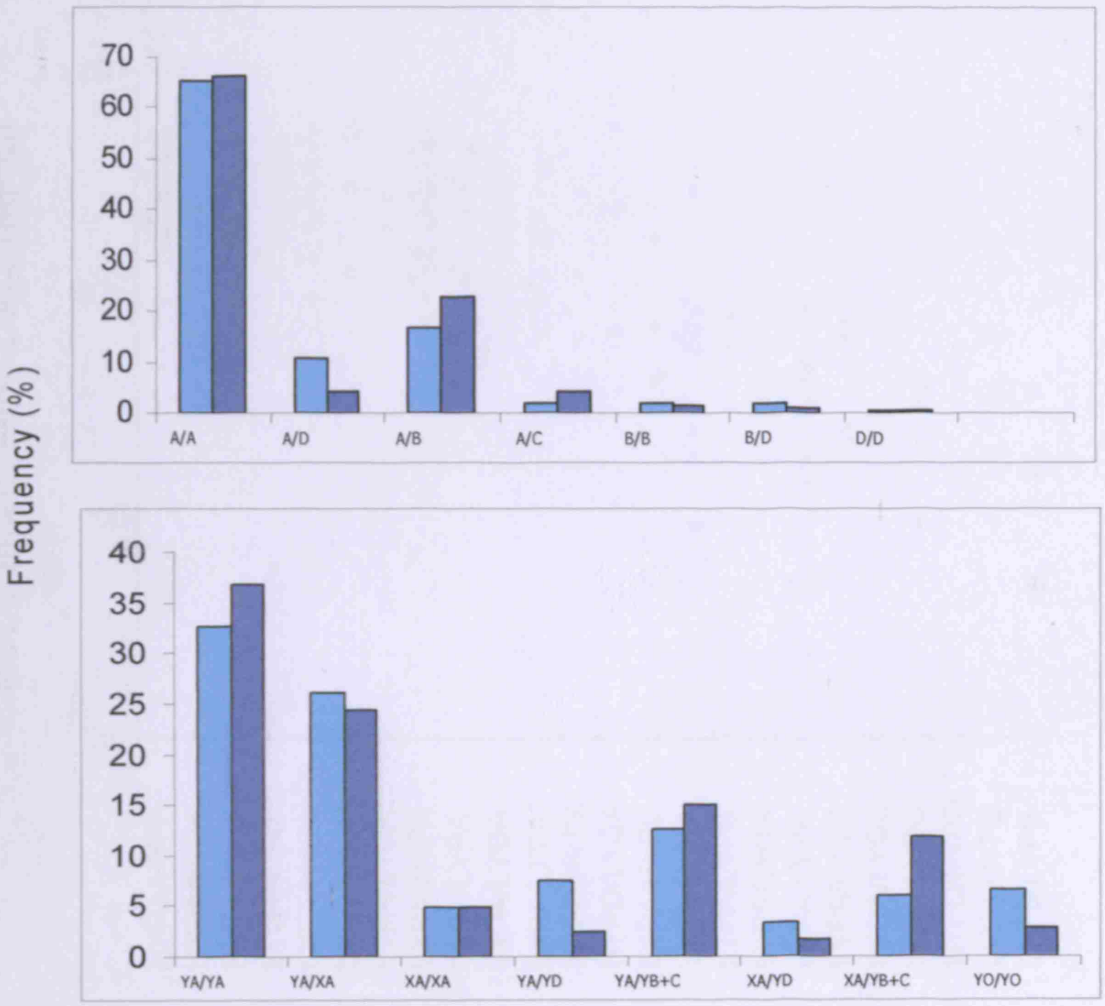


Figure 5-8. The distribution of MBL haplotypes in a cohort of cystic fibrosis patients and an unselected control population ALSPAC.

Turquoise bars represent control samples (Children in Focus from ALSPAC study), and dark blue bars indicate patients with cystic fibrosis cohort (n=249).

5.3.3.3 Correlation of haplotype and levels

There was a significant correlation between serum MBL level and haplotype (Figure 5-9; $p<0.001$). The highest protein levels were seen in patients with wild type structural alleles and the lowest values were observed in those individuals homozygous or compound heterozygous for structural mutations, and in heterozygotes with the X promoter mutation (Figure 5-9).

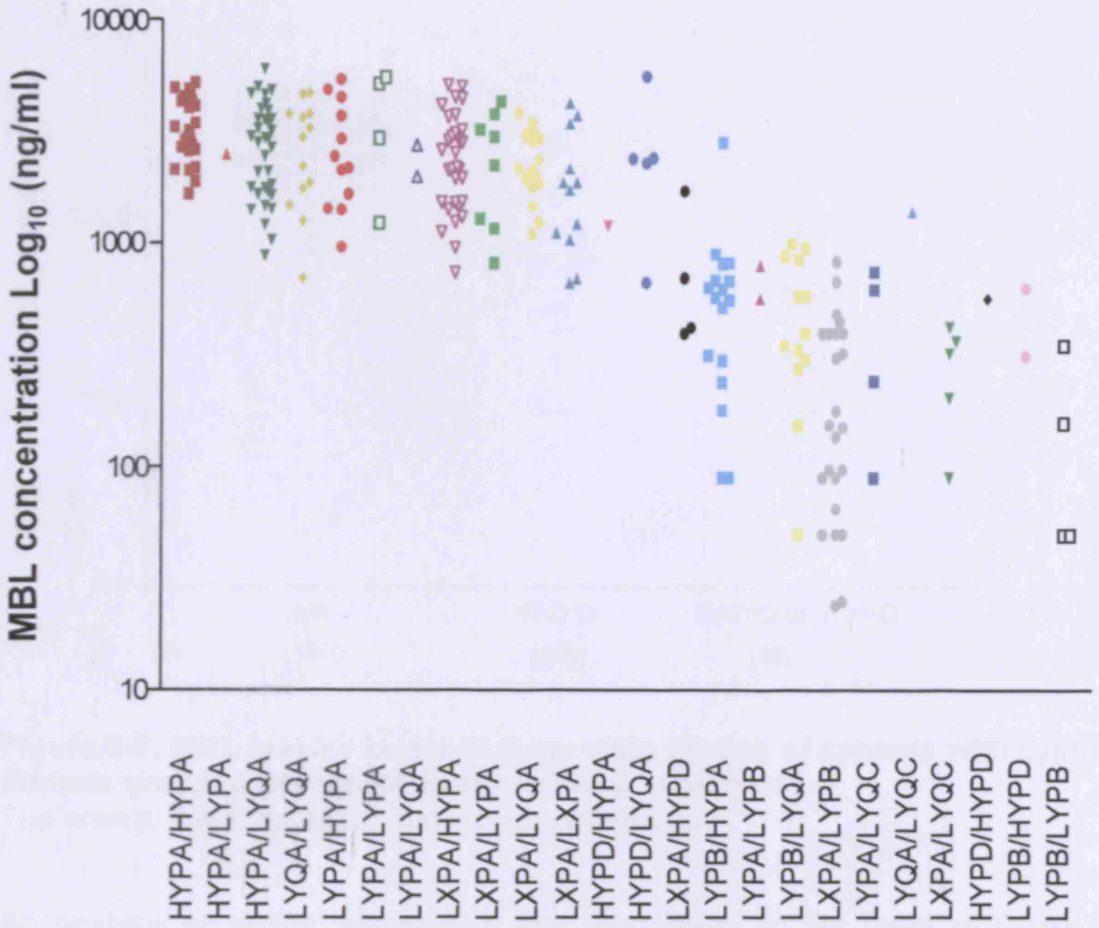


Figure 5-8. The correlation between serum MBL levels and MBL2 haplotypes in children with cystic fibrosis.

Post hoc analysis revealed three significantly different haplotype groups based on protein levels: group 1 A/A (with either promoter polymorphism); group 2 YA/O; and group 3 XA/O and O/O (Figure 5-9).

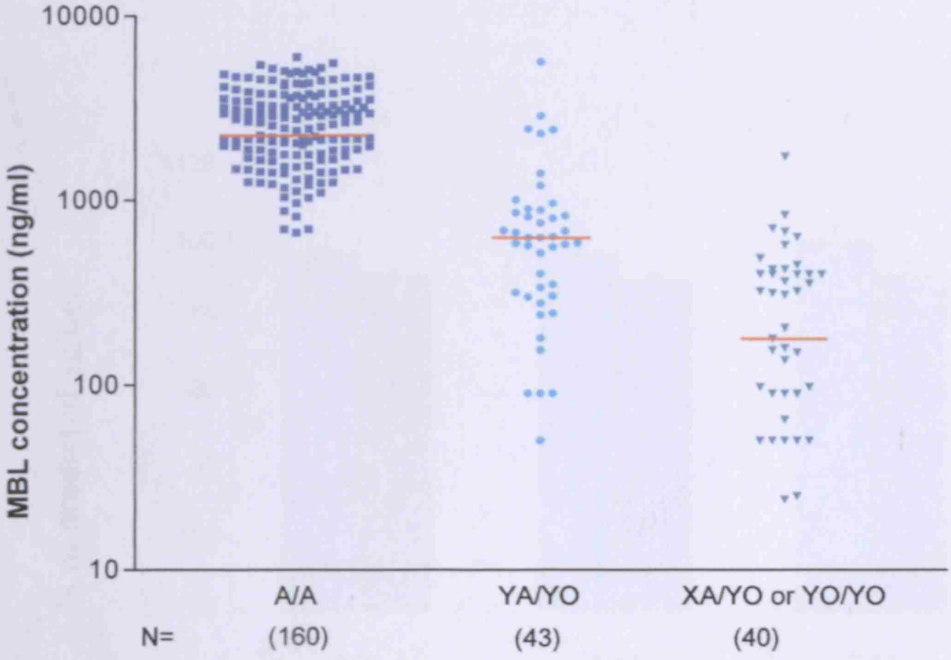


Figure 5-9. MBL plasma levels in three main groups of patients with cystic fibrosis grouped according to short MBL2 haplotype.
The orange lines represent median for each group.

An analysis of clinical parameters was then made on the basis of these three groups. Levels were similar in control and cystic fibrosis groups, with the exception that the ALSPAC control children with wild type alleles had significantly ($p<0.001$) higher levels (Mean = 3929 ng/ml) than those observed in children with cystic fibrosis (Mean=2748 ng/ml).

5.3.3.4 Lung function

A total of 191 children had their lung function measured on at least one occasion, with the others being too young to perform the manoeuvre. As none of the O/O children was > 12 years old, all comparisons made for lung function were obtained for children between the ages 7-11 years (mean age A/A: 9.7± 0.2 years, A/O: 9.7±0.2 years, O/O: 8.6±1.0 years; ANOVA p=3). Neither mean FEV1 not FVC was reduced at this stage in children possessing the O/O haplotype (Fig. 5-10), and there was no effect of the X-promoter polymorphism.

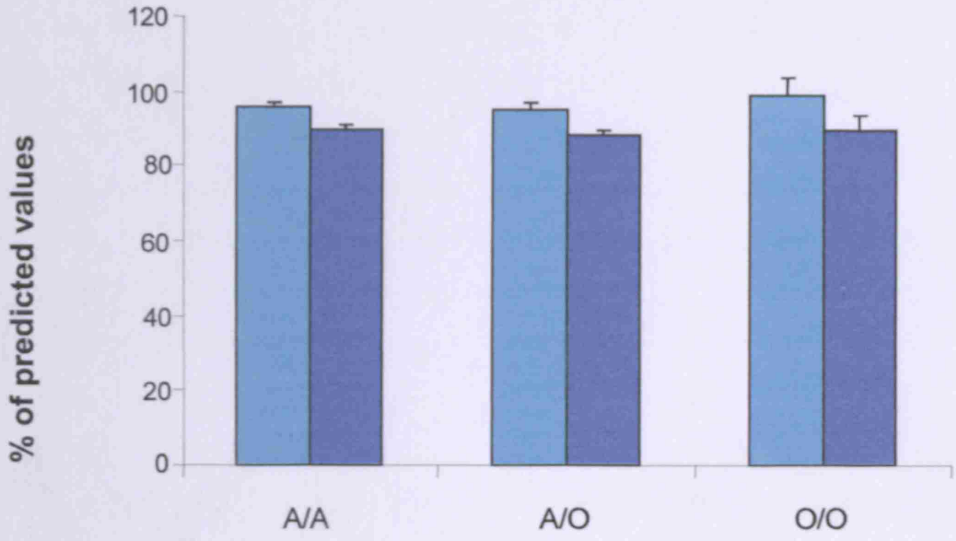


Figure 5-10. The effect of mannose-binding lectin genotype on lung function in patients with cystic fibrosis.

There was no significant effect of MBL on FEV1 (light blue bars) and FVC (dark blue bars) between the ages 7-11 years (all recordings are presented as means for each individual). A/A: n=113; A/O: n=67; O/O: n=7.

5.3.3.5 Infection and Inflammation

In total, 85.1% of children had experienced at least one positive culture for *P.aeruginosa*, and, in 28.8%, the organism had a mucoid phenotype suggestive of chronic infection. For the group as a whole, infection with *P.aeruginosa* before the first lung function measurement had a significant adverse effect on both FEV_1 (87.2±1.7% versus 96.1±2.2%; $p<0.01$) and FVC (95.2±1.4% versus 101.3±2.1%; $p=0.02$). However, there was no association between infection rates, age or mucoid conversion and MBL2 haplotype. By the end of this study, seven children had produced at least one positive culture for *B. cepacia*. There was no apparent trend with regard to MBL haplotype and number of positive cultures (YA/YA: n=3,

YA/XA: =2, YA/O: n=1, XA/O: n=1). No differences were observed between MBL2 haplotype groups with regard to ever having been admitted for *i.v.* antibiotics or age at first admission.

5.3.4 Discussion

This study investigated the influence of MBL on lung function in the largest group of children with CF reported to date. Low MBL levels were not obviously associated with poor pulmonary function during childhood. Moreover, there was no significant difference in percent predicted FEV_1, FVC or annual rate of decline between high, medium and low-expressing MBL haplotype groups.

These findings differ from the three previous published studies in adults. Davies *et al.* reported that adults possessing two abnormal structural MBL2 alleles have significantly reduced lung function, lower oxygen saturations and increased requirements for hospital admission. Gabolde *et al.* matched 11 MBL-deficient adults (O/O) with homozygous sufficient controls (A/A), and reported that MBL deficiency led to significantly worse lung function. No disadvantage was seen in heterozygotes. In contrast, Garred *et al* reported that either one or two structural MBL2 gene mutations (A/O or O/O) led to the impairment of FEV_1 and reduced survival.

The reasons for the differences between adults and children are unclear. However, it is recognised that the group of O/O children in this study was very small (n=7), and thus may have been underpowered to detect a difference in lung function. This is a recurring problem in modifier-gene studies attempting to examine the effect of fairly rare genotypes. In addition, it is likely that any adverse effects may be more difficult to detect in the younger age group before so much lung injury has occurred. Whilst Garred *et al.* (1999) observed an effect of MBL in individuals as young as 8 years old, most of the children in this study had well-preserved lung function, and low or undetectable levels of systemic inflammatory markers.

Overall Conclusion

This chapter reports three cohorts of patients with three different diseases caused by or exacerbated by infectious agents. In the two studies in which MBL appeared to have an influence on the disease process, the explanation remains unclear. Many studies have focussed on the susceptibility of individuals to an infectious agent. However in all of these three cohorts there was little evidence that this was the main effect of MBL. A more likely explanation appears to be that MBL is able to modify the host response to infection and this concept will be further explored in the next chapter.

Chapter 6 MBL influence on endothelial cells

6.1 Introduction

Capillary leak and intravascular thrombosis are serious consequences of meningococcal sepsis and are indicative of widespread vascular endothelial injury (Mercier *et al.*, 1988). Histological studies of meningococcal disease show that cutaneous lesions contain large numbers of organisms associated with the vascular endothelium (Hill and Kinney, 1947). Recent studies have also shown that meningococci have the capacity to bind to endothelial cells in a receptor-ligand specific fashion (Virji *et al.*, 1993; Virji *et al.*, 1992) and indicate that bacterium-endothelial contact may itself be critical in mediating the vascular injury seen in this disease (Sotto *et al.*, 1976). There is evidence that meningococci, both alone and in the presence of neutrophils, can lead to endothelial damage (Klein *et al.*, 1996; Virji *et al.*, 1991).

Expression of adhesion molecules by the vascular endothelium is a critical step in the inflammatory response. Leukocyte adhesion occurs through a complex and multistep process involving initial tethering and then rolling of leukocytes by low-avidity interactions, mainly with the selectin family of cell adhesion molecules (e.g., CD62E/E-selectin). This is followed by firmer adhesion, which is mediated largely by higher-affinity interactions involving the members of the immunoglobulin Ig superfamily (e.g., ICAM-1 and VCAM-1) on endothelial cells (Springer, 1994). After firm adhesion, transendothelial and subendothelial migration may occur, a process also involving leukocyte integrins and complex cross talk among leukocytes, the endothelium, cytokines, and chemokines.

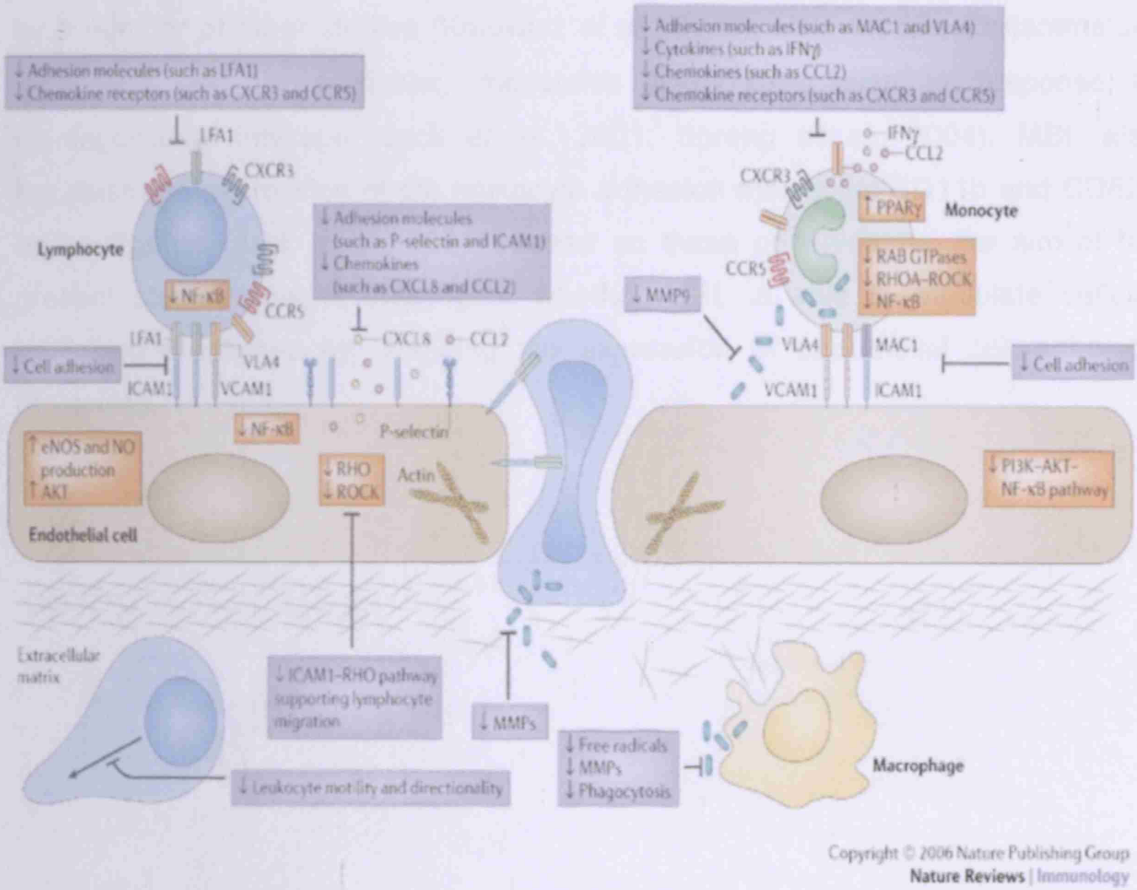


Figure 6-1. The expression of adhesion molecules by endothelial cells and leukocytes.

Taken from Nature Reviews (2006).

The initial inflammatory stimulus to this activation cascade is critical since it determines which leukocytes will participate in the subsequent inflammatory response (Carlos and Harlan 1994). CD62E is expressed solely on vascular endothelium, and has a significant role in recruitment of both neutrophils and monocytes in the early phase of the inflammatory response (Lawrence, Bainton & Springer 1994; Lawrence & Springer 1993). This is consistent with the time course of surface CD62E expression in response to inflammatory stimuli in vitro. It appears at around 1 hour, peaks at 4 to 6 hours and disappears by 24 hours (Bevilacqua *et al.*, 1987). Zimmernan *et al.* (1992) proposed that sustained adhesion of neutrophils is dependent on expression of CD62E that is transcriptionally up-regulated in response to pro-inflammatory cytokines such as IL-1 and TNF-α and LPS (Zimmerman, Prescott & McIntyre 1992). This is supported

by a number of other studies (Kotowicz *et al.*, 2000). MBL modifies inflammatory responses and, in particular, modulates IL-1β expression in response to meningococcal infection (Jack *et al.*, 2001; Sprong *et al.*, 2004). MBL also regulates the expression of the leukocyte adhesion molecules CD11b and CD62L on neutrophils (Jack *et al.*, 2001). Based on these observations, the aim of the present studies was to investigate whether MBL is able to modulate cellular trafficking processes by changing the expression of endothelial cell adhesion molecules.

6.2 Materials and Methods

6.2.1 Buffers

Puck's A saline	KCl	0.4 g/L
	NaCl	8.00g/L
	$NaHCO_3$	0.35 g/L
	glucose	1.0 g/L
	EDTA	0.2%
	FCS	10%
	phenol red	0.005g/L
Phosphate buffered saline	NaCl	140.0mM
	KCl	2.7mM
	Na_2HPO_4	8.0mM
	KH_2PO_4	1.5mM
One tablet of PBS was added to 100ml of ddH_2O and autoclaved		
FACS washing buffer	1 times PBS	
	0.02% Sodium azide	
	5% FCS	

6.2.2 Endothelial Cell Culture

6.2.2.1 Background

Endothelial cells can be isolated and cultured from a wide variety of sources, including different anatomical sites and different species. Each type requires specific techniques for their isolation and growth. In particular, they may require various growth factors and special media. In general, endothelial cells require media which are rich in amino acids and various sugar moieties. They also require high quality fetal calf serum at high concentration (20%).

Human umbilical vein cells are an ideal source of endothelial cells since they are derived from a plentiful resource that would otherwise be discarded. The basic method employed was as described by Kotowicz *et al.* (1996).

6.2.2.2 HUVEC Culture media

RPMI 1640 medium, containing 10mM L-glutamine, 80μg/ml gentamicin, 100 units penicillin/streptomycin was used for the collection and storage of umbilical cords, and kept at 4°C in autoclaved polypropylene bottles. Washing of umbilical cords was performed with RPMI 1640 with the same supplements but containing 5% fetal calf serum (FCS) (HUVEC wash medium).

1% Collagenase type II solution, comprising 1g collagenase dissolved in 1L of Dulbecco's Modified Eagle Medium, which was then passed through a 0.2 micron tissue culture filter and stored in aliquots at –20°C until required.

Primary and secondary cultures used a specialised medium for growth of endothelial cells, MCDB 131, supplemented with 10mM L-glutamine, 100 u/ml penicillin/streptomycin and 20% fetal calf serum.

6.2.2.3 Fetal calf serum

The quality and batch of FCS was found to be a critical determinant of yield of HUVEC from either primary culture or growth in secondary culture. For this reason, batches of FCS were tested for quality. Specific batches, which proved to be most effective at sustaining growth of HUVEC, were ordered in large quantities and were used solely for HUVEC culture. Fetal calf serum was heat inactivated at 56° C for 60 minutes and stored in aliquots at -20^0 C until required.

6.2.2.4 Protocol for Isolation of HUVEC

Only fresh, intact cords were used, usually within 72 hours of collection. Cords that were meconium stained were discarded. All the procedures described below were performed in a Class II safety cabinet using aseptic techniques and autoclaved glassware. All instruments were sprayed with 70% IMS after thorough cleaning. Culture and wash media and enzymes were warmed to 37° C in a water bath.

The cords were inspected for damage such as cuts and needle punctures from cord blood sampling. Suitable cords were then sprayed with 70% IMS and blood

expressed into a collection pot. Washed cords were then placed in a Duran bottle containing RPMI 1640 medium with added antibiotics as described above.

Single cords were then cut at least 1cm from both ends. One end was clamped with an artery forceps. The other end was then inspected and the vein identified. The vein was dilated with blunt forceps, and cannulated with a plastic filling tube. This filling tube was secured with cotton thread and finally a small artery forceps. The vein was then filled with warmed RPMI 1640 medium with 5% FCS (wash medium). Sites of leakage were clamped if necessary. The vein was flushed with wash medium to remove excess blood, and then filled with 1% Collagenase II solution. The cord was then incubated at 37°C in 5% CO_2 for 20 minutes. After this incubation, the digest was transferred from the cord into sterile Duran bottle, and flushed through with an equal volume of wash medium. This was transferred into a sterile, 50ml conical tube. The digest was centrifuged at 200g for 7 minutes at room temperature. The supernatant was discarded, and the pellet resuspended in MCDB 131 medium with 20 % FCS (HUVEC culture medium).

6.2.2.5 HUVEC primary culture

For tissue culture the digest was transferred to 25 cm^2 surface modified polystyrene flasks. These were incubated at 37° C in a 5% CO_2 atmosphere Cells were washed in fresh culture medium on the following day and inspected under phase contrast microscopy.

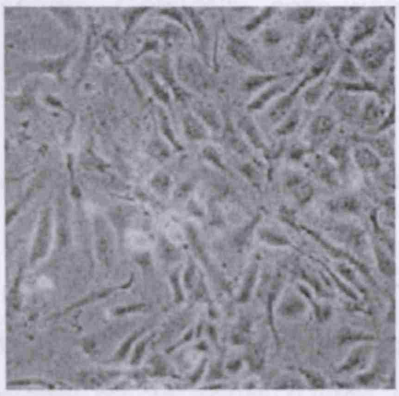


Figure 6-2. HUVEC preparations viewed under the inverted microscope.

HUVEC could be identified as small clusters of oval adherent cells. Cultures were inspected each day for growth. When cells were approaching confluence, which

usually occurred after 72 hours in culture, they were subcultured. Cells, which grew poorly, were observed to be contaminated with smooth muscle, or fibroblastic cells were discarded.

6.2.2.6 HUVEC subculture

HUVEC that were near confluent were washed three times in warmed PBS or HBSS to remove non-adherent cells and protein in FCS. Cells were washed once in 0.5 ml Trypsin-EDTA solution (pre 25 cm^2 flask), removed and a further 0.5-ml Trypsin-EDTA solution added.

Cells were inspected under phase contrast microscopy and when cells were rounding and becoming dislodged (usually after 30 seconds), the flask was tapped against the bench top to aid removal. The cells were quickly resuspended in warmed HUVEC culture medium and transferred to a 50-ml sterile conical tube. These were then seeded into 24 well flat bottom tissue culture plates which had been surface treated with endothelial cell attachment factor. Tissue culture plates were then incubated at 37° C in 5% CO_2.

6.2.2.7 Use of HUVEC in experiments

HUVEC morphology and phenotype alter dramatically after serial passages especially in terms of induction of cell adhesion molecules in response to pro-inflammatory stimuli. The cells used throughout this study were always obtained after the first subculture passage. Generally, cells were used within one week of passaging. Medium was replaced every 3 days.

6.2.2.8 Protocol for detection of cell adhesion molecule expression by flow cytometry

The study by Dixon *et al.* (1999) showed that with $5x10^6$ CFU/ml *N.meningitidis* B1940, CD62E expression by endothelial cells reached maximum levels at 5 hours, and diminished markedly by 24 hours (Dixon *et al.*, 1999). ICAM-1 was expressed on resting HUVECs, but the levels were seen to increase by 4 hours and were still rising by 24 hours (Dixon *et al.*, 1999). In the present study MBL at a concentration of 5 μg/ml was added to some wells containing HUVEC. The endothelial cells were then stimulated with meningococci prepared at

concentrations of $5x10^6$ and $5x10^5$ for 5 and/or 24 hours at 37°C in a 5% CO_2 atmosphere. After stimulation, medium from all wells of a 24 well plate was removed and discarded. Each well was washed once in warmed Puck's A saline and then incubated in 0.5ml Puck's A saline for 15 minutes at 37° C on a rocking platform. Cells were removed by mechanical scraping with a Pasteur pipette, and transferred to 12 x 75mm FACS tubes in 2 ml of FACS wash (see Section 6.2.1). Cells were divided into an appropriate number of separate tubes (one tube per stain), and centrifuged at 200g for 5 minutes. Each sample was incubated with monoclonal antibody to CD62E, and ICAM-1 for 15 minutes at room temperature. An isotype matched IgG1 control was included to control for non-specific binding of antibodies. Cells were washed in a 2 ml wash volume and centrifuged at 200g for 5 minutes. Samples were incubated with goat anti-mouse F $(ab')_2$ phycoerythrin (PE) conjugated antibody for 15 minutes in the dark at room temperature. After final centrifugation, cells were resuspended in 0.3 ml Cellfix solution until flow cytometry was performed.

6.2.2.9 Flow Cytometry

Fixed endothelial cells were analysed on a FACSCalibur cytometer using CellQuest software. Typical size and granularity characteristics and also the presence of CD31, as shown in Figure 6-3, confirmed the identity of endothelial cells. 5000 events within the endothelial gate were collected. Typical instrument settings for endothelial cells are given in Appendix II.

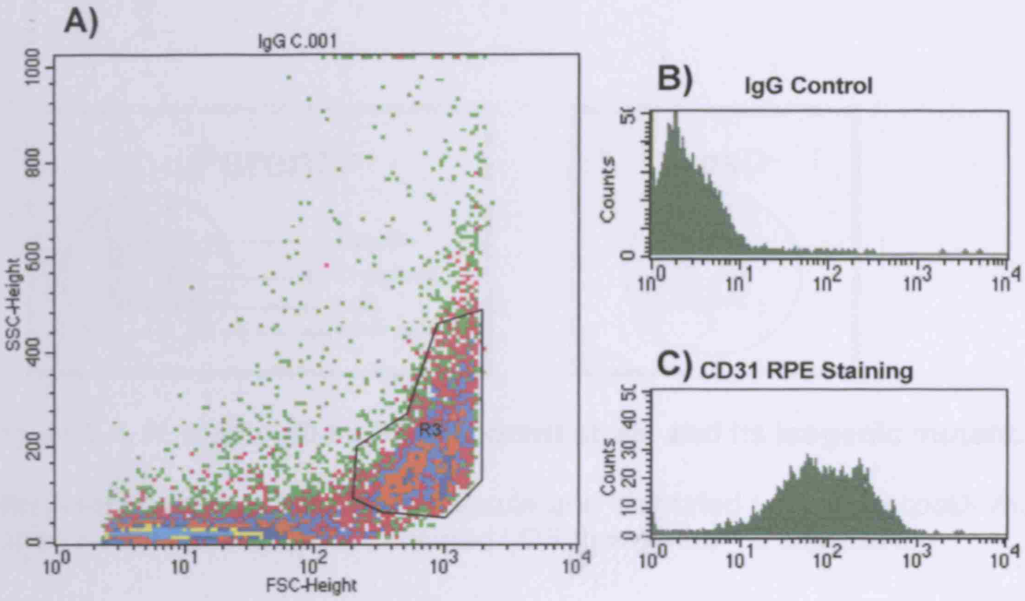


Figure 6-3. Flow cytometric characteristics of HUVEC.

Endothelial cells were identified by forward and side scatter characteristics and gated accordingly. Staining of an irrelevant isotype matched control and antibody raised against the endothelial specific surface marker CD31 (PECAM) are shown.

6.2.3 Microbiological Techniques

6.2.3.1 Bacterial strains

The parent organism *N. meningitidis* B1940 and its mutant were a gift from Professor Matthias Frosch, Institute of Hygiene and Microbiology, Wurzburg, Germany. Both have been described previously (Hammerschmidt *et al.*, 1994). The parent organism B1940 expresses capsule and possesses a lipooligosaccharide (LOS) of immunotype L3 that can be fully sialylated (Figure 6-3). The *cpsD*- mutant is capsulated and has an inactive *galE* gene, which codes for the enzyme UDP-epimerase. This mutant cannot add a galactose moiety to glucose at the start of the α-oligosaccharide chain of LOS. The resultant LOS is therefore truncated, and does not have the galactose acceptor site for sialic acid. It is therefore non-sialylated (see Figure 6-4).

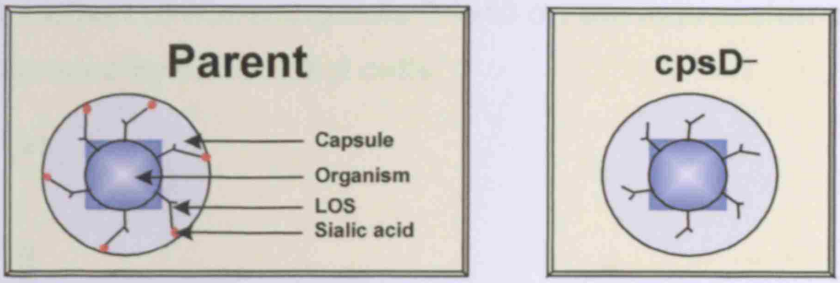


Figure 6-4. *N. meningitidis* B1940 parent strain and its isogenic mutant.

The parent organism possesses capsule and sialylated LOS. The *cpsD*- mutant is capsulated but possesses a truncated LOS that cannot be sialylated.

Despite these changes, all the organisms express pili and the Class V outer membrane proteins Opa and Opc (Klein *et al.*, 1996).

6.2.3.2 Growth and Preparation of *Neisseria meningitidis*

The organisms were grown as described in Chapter 3.2.

6.3 Results

6.3.1 Effect of *N.meningitidis* B1940 on the expression of adhesion molecules by endothelial cells

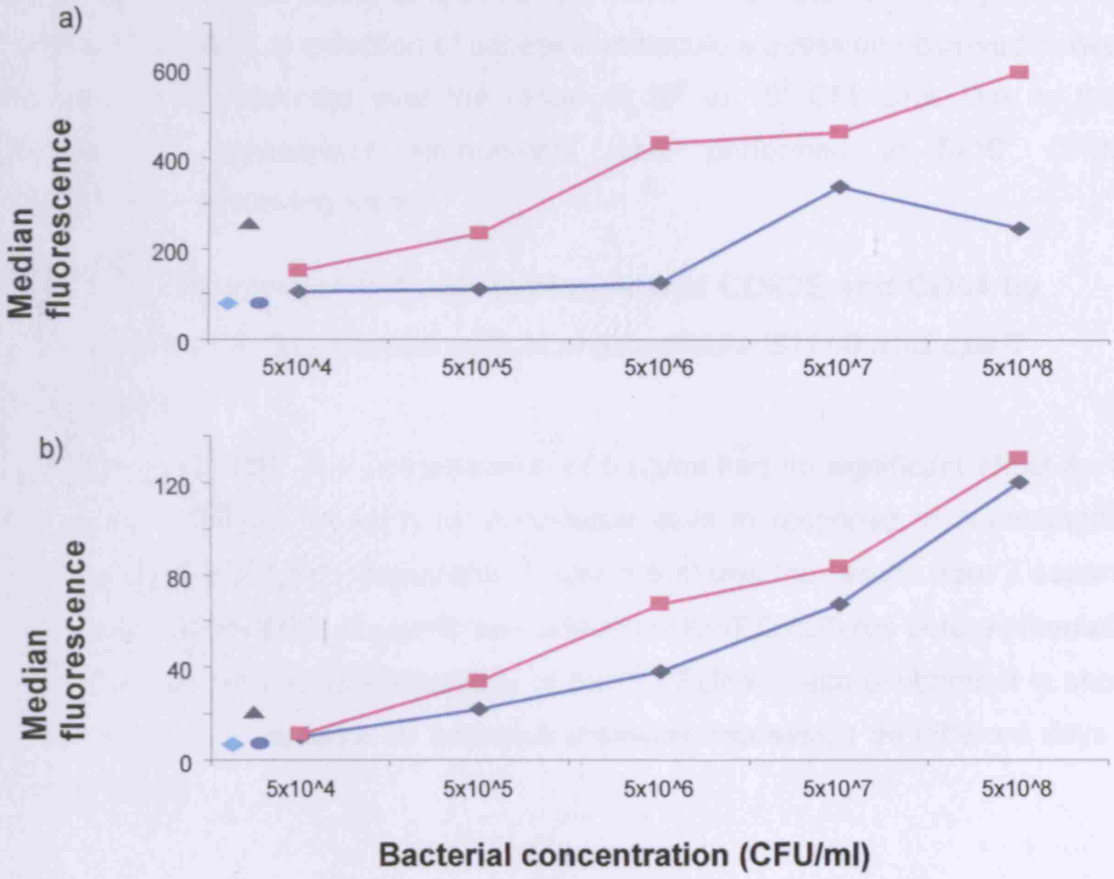


Figure 6-5. The effect of bacterial concentration of B1940 parent and *cpsD*-mutant on the induction of CD62E and CD54 (ICAM1) expression after 5 hours of exposure.

HUVEC were exposed to various concentrations of organisms in 5 hour incubations prior to determination of cell adhesion molecule expression. The blue line represents the experiment with *N.meningitidis* B1940 wild type and the pink colour line represents the experiments with *cpsD*- mutant organism. The blue circle represents the expression of adhesion molecules by HUVEC with medium alone, the turquoise diamond – the same results were obtained when MBL was added to medium at a concentration of 5 μg/ml. The navy triangle represents the expression of CD54 and CD62E upon stimulation of endothelial cells with meningococcal LPS at a concentration of 10 ng/ml. Panel (a) represents the expression of CD54 adhesion molecule by HUVEC, and Panel (b) represents the expression of CD62E molecule by endothelial cells. This is representative of 3 separate experiments each of which yielded similar results.

After 5 hours incubation with fixed *N.meningitidis* B1940 and its isogenic mutant organism, *cpsD-*, an increase in CD62E and ICAM1 staining was detected. The expression of both adhesion molecules was greater with increasing concentrations of bacteria. Figure 6-5 shows that the unencapsulated *cpsD-* mutant with truncated LOS induced higher levels of CD62E and ICAM1 than the parent organism. The largest differences in induction of adhesion molecule expression observed between the two strains occurred over the range of 10^5 to 10^7 CFU's/ml. Due to these findings the subsequent experiments were performed at $5x10^6$ CFU/ml concentration of meningococci.

6.3.2 The influence of MBL on expression of CD62E and CD54 by endothelial cells incubated with *N.meningitidis* B1940 and *cpsD-* organisms

The addition of MBL at a concentration of 5 μg/ml had no significant effect on the expression of CD54 (ICAM1) by endothelial cells in response to *N.meningitidis* B1940 parent and *cpsD-* organisms. Figure 6-5 shows the results from 7 separate experiments when MBL (5 μg/ml) was added to HUVEC cultures before stimulation using both bacteria at concentrations of $5x10^6$ CFU/ml. Each experiment is shown separately due to variation in adhesion molecule expression on different days by different cells.

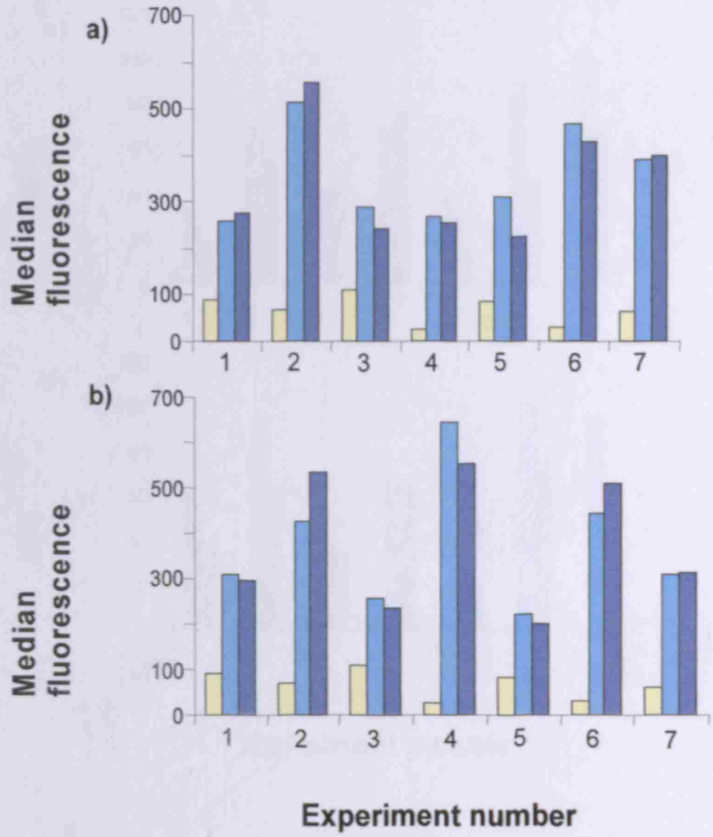


Figure 6-6. Effect of MBL on HUVEC expression of CD54 induced by *N.meningitidis* B1940 .

N.meningitidis B1940 parent (panel a) and *cpsD*- organisms (panel b) at a concentration of $5x10^6$ were added to HUVEC preincubated with MBL (5 μg/ml). Cell adhesion molecule expression was determined after 5 hours of incubation. The yellow bar represents CD54 expression without stimulation, the turquoise bar demonstrates the expression of CD54 after stimulation of endothelial cells with bacteria alone and the blue bar shows the results of experiments with added MBL.

In contrast to CD54, there was a consistent and significant decrease in CD62E expression by endothelial cells in response to the addition of 5 μg/ml of MBL prior to incubation with both organisms. Figure 6-6 shows the results from 7 separate experiments when endothelial cells were incubated for 5 hours with *N.meningitidis* B1940 parent and *cpsD*- organisms at a concentration of $5x10^6$ CFU/ml.

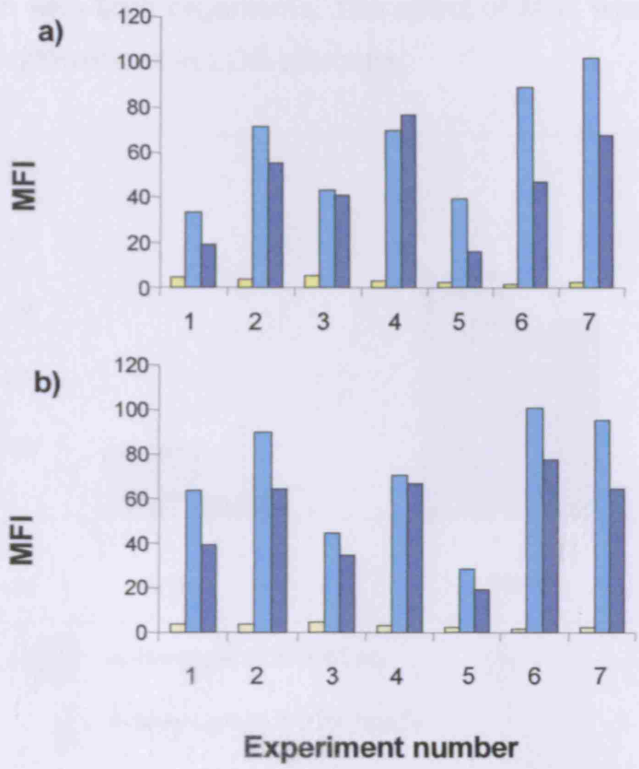


Figure 6-7. Effect of MBL on HUVEC expression of CD62E induced by *N.meningitidis* B1940.

(a) *N.meningitidis* B1940 parent and (b) *cpsD*- organisms at a concentration of $5x10^6$ were added to HUVEC preincubated with MBL (5 μg/ml). Cell adhesion molecule expression was determined after 5 hours of incubation. The yellow bar represents CD62E expression without stimulation, the turquoise bar demonstrate the expression of CD62E after stimulation of endothelial cells with bacteria only and the blue bar shows the results of experiments with added MBL.

In order to compare the results between separate experiments, the percentage change in CD54 and CD62E expression by endothelial cells upon stimulation with *N.meningitidis* B1940 and the *cpsD*- mutant in the presence and absence of MBL was calculated and the results are shown in Figure 6-8. The change in median fluorescence for CD54 in the presence of MBL following incubation with *N.menigitidis* B1940 parent and *cpsD*- organisms did not change significantly ($p>0.05$, two-sided Student's t-test). In contrast the presence of MBL at 5 μg/ml significantly ($p<0.05$) reduced the expression of CD62E by HUVEC upon

stimulation with both organisms. The effect of MBL was similar for both organisms in spite of differences in LOS structure.

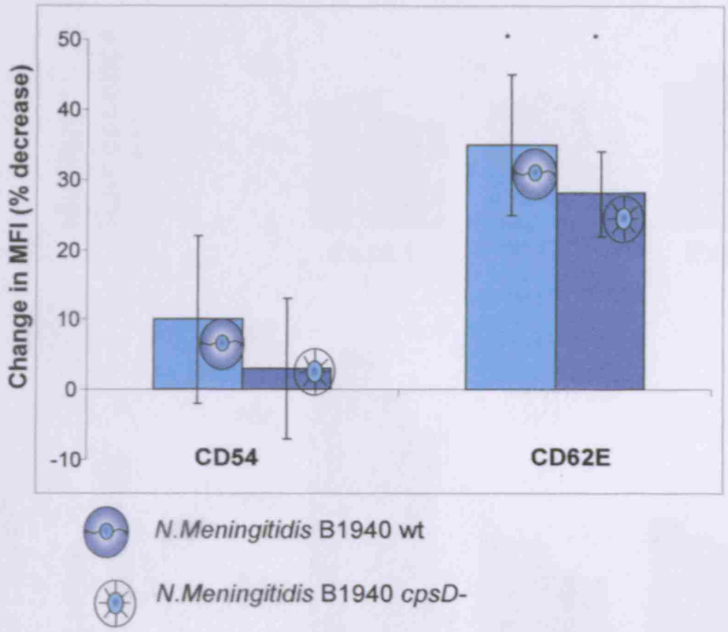


Figure 6-8. Effect of MBL on expression by endothelial cells of CD54 and CD62E in response to *N.meningitidis* B1940 wild type and *cpsD-*.

N.meningitidis B1940 parent and *cpsD-* organisms at a concentration of $5x10^6$ CFU/ml were added to HUVEC preincubated with 5 μg/ml MBL. Cell adhesion molecule expression was determined after 5 hours of incubation. The results are expressed as mean change in median fluorescence from three separate experiments. Error bars representing the 95 % confidence intervals are shown.

The effect of duration of exposure to meningococcal strains on HUVECs incubated over a longer period of time in the presence or absence of MBL was studied. Figures 6-9 and 6-10 show the results of experiments when HUVECs were exposed to the microorganisms for 5 and 24 hours. Longer exposure to both strains did not consistently influence the effect of MBL on CD54 expression. However the effect of MBL on CD62E expression was abrogated by these longer exposures (Figure 6-11).

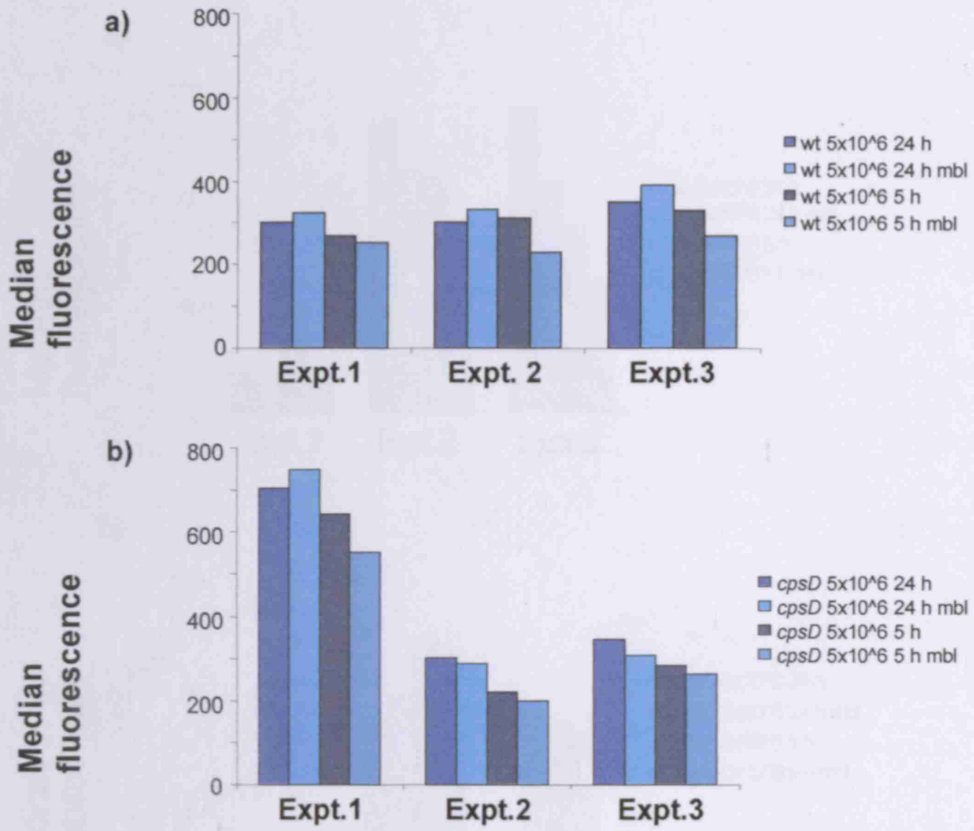


Figure 6-9. Effect of MBL on *N.meningitidis* B1940 induced HUVEC expression of CD54 after different incubation times.

N.meningitidis B1940 parent (panel a) and *cpsD*- organisms (panel b) at a concentration of $5x10^6$ were added to HUVEC preincubated with 5 μg/ml MBL. Cell adhesion molecule expression was determined after 5 and 24 hours of incubation. The results of three different experiments are shown.

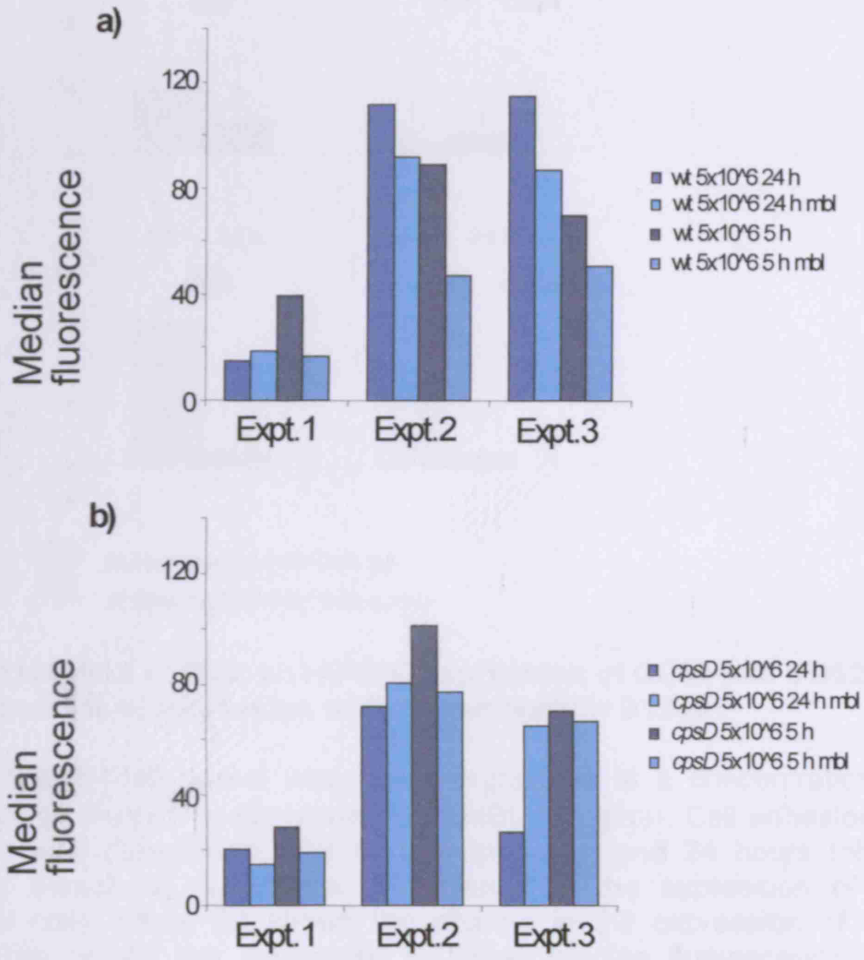


Figure 6-10. Effect of MBL on HUVEC expression of CD62E induced by *N.meningitidis* B1940.

N.meningitidis B1940 parent (panel a) and *cpsD*- organisms (panel b) at a concentration of $5x10^6$ were added to HUVEC preincubated with 5 μg/ml MBL. Cell adhesion molecule expression was determined after 5 and 24 hours of incubation.

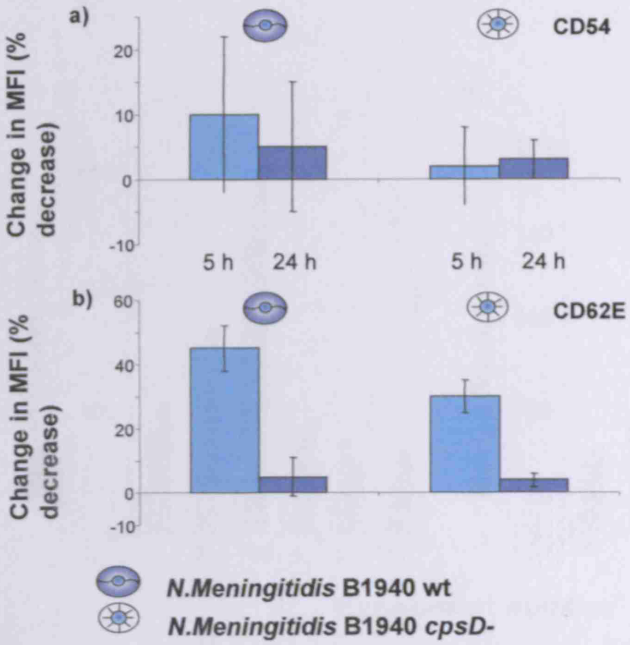


Figure 6-11. Effect of MBL on HUVEC expression of CD54 and CD62E after different periods of incubation with *N.meningitidis* B1940 .

N.meningitidis B1940 parent and *cpsD-* organisms at a concentration of $5x10^6$ were added to HUVEC preincubated with MBL (5 μg/ml). Cell adhesion molecule expression was determined after 5 (turquoise bar) and 24 hours (blue bar) of incubation. Panel (a) represents the change in the expression of CD54 by endothelial cells; panel (b) shows the change in the expression of CD62E by HUVEC. The results are expressed as mean median fluorescence from three separate experiments. Error bars representing the 95 % confidence intervals are shown.

6.3.3 The influence of MBL on CD62E and CD54 expression by endothelial cells in response to LPS

The addition of MBL at a concentration of 5 μg/ml did not significantly change the expression of CD54 (ICAM1) and CD62E by endothelial cells in response to meningococcal LPS after 5 hours of incubation ($p>0.05$). Figure 6-12 shows the results of 6 separate experiments. Each experiment is shown separately due to variation in adhesion molecule expression on different days by different cells.

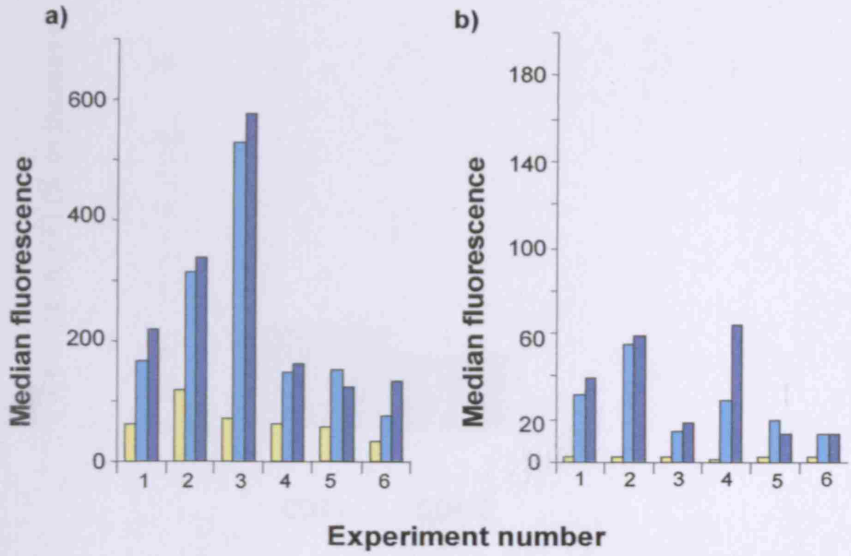


Figure 6-12. Effect of MBL on *N.meningitidis LPS* induced HUVEC expression of CD54 and CD62E.

N.meningitidis B1940 LPS at a concentration of 10ng/ml was added to HUVEC preincubated with 5 μg/ml MBL. Cell adhesion molecule expression was determined after 5 hours. Panel (a) shows the expression of CD54 upon stimulation in 6 independent experiments and panel (b) shows the expression of CD62E in the same experiments. The yellow bar represents expression without stimulation, the turquoise bar shows the expression after stimulation of endothelial cells with LPS only, and the blue bar shows the results of experiments with added MBL.

Figure 6-13 shows the change in percentage of CD54 and CD62E expression by endothelial cells in response to LPS stimulation in the presence and absence of MBL. Incubation for 24 hours did not change the influence of MBL on expression of both adhesion molecules by HUVEC in response to bacterial LPS.

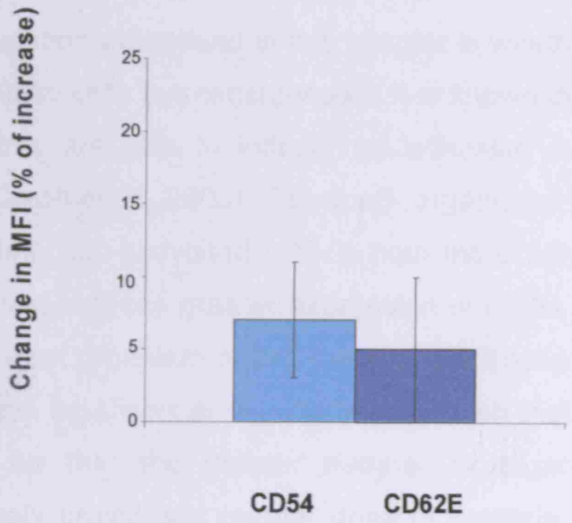


Figure 6-13. Effect of mannose-binding lectin on expression of CD54 and CD62E by endothelial cells in response to meningococcal LPS

N.meningitidis B1940 LPS at a concentration of 10ng/ml was added to HUVEC preincubated with MBL (5 μg/ml). Cell adhesion molecule expression was determined after 5 hours of incubation. The results are expressed as mean median fluorescence from three separate experiments. Error bars representing the 95 % confidence intervals are shown.

6.4 Discussion

The question addressed in this chapter is whether MBL modulates the activation of endothelial cells by meningococci. It is known that meningococci with different LPS structures are able to induce cell adhesion molecule expression on endothelial cells (Dixon *et al.*, 2000). The *cpsD-* organism of *N.meningitidis* B1940 which has a truncated, non-sialylated LPS is both more adherent to endothelium (Klein *et al.*, 1996) and induces greater expression of CD54 and CD62E molecules by HUVEC than parent organism of the same strain. These data correspond to the findings of the study by Dixon *et al.* which suggested that a reasonable explanation for this would be that the greater number of organisms bound to endothelial cells effectively provides a greater dose of bacteria (and hence inflammatory moieties such as LPS) to stimulate HUVEC (Dixon *et al.*, 2000).

The concentration of 5 μg/ml of mannose-binding lectin used in these experiments resulted in an average of 38% and 30% inhibition of CD62E expression by endothelial cells in response to parent *N.meningitidis* B1940 and its *cpsD-* organisms respectively. The presence of the lectin at the same concentration did not have any significant effect on the expression of CD54 in response to both microorganisms.

Endothelial expression of CD54 in vitro peaks by 12 hours and persists for at least 72 hours (Pober *et al.*, 1986; Rice *et al.*, 1989). In contrast CD62E is at its highest after 4 hours post stimulation with a return to basal level of expression within 24 hours (Bevilacqua *et al.*, 1987). Because of this, the effect of MBL was explored after different periods of incubation. MBL still did not have any significant effect on CD54 expression after 24 hours stimulation. The expression of CD62E was low by this time but little effect of MBL was observed.

MBL binds avidly to the *cpsD-* mutant but not to the sialylated parent strain (Jack *et al.*, 1998). However, its binding properties did not appear to influence the inhibitory effect of the lectin on the expression of CD62E. In the study by Jack *et al.* (1998) flow cytometry was used to assess MBL binding to *N.meningitidis* B1940. Two other studies (Jack *et al.*, 2001; Sprong *et al.*, 2004) have demonstrated by

electron microscopy and dot blotting techniques that there is a low level binding of MBL to microorganisms which is non-detectable by flow cytometry.

It is not known how much MBL binding is required for an effect on CD62E expression. Experiments were not performed with lower concentrations of MBL and were carried out in the absence of complement. It is likely therefore that MBL acts via a receptor or group of receptors that can modulate CD62E expression on endothelium. Several putative candidate MBL receptors have been proposed in the literature (Eggleton *et al.*, 1998). One of these is designated C1qRp and was recently identified as CD93 (Steinberg *et al.*, 2002). It is abundant on endothelial cells, (our unpublished data) and has been considered to be an MBL receptor (Tenner *et al.*, 1995). However, data indicate that C1qRp/CD93 may function by modulating phagocytic activity via regulation of adhesion and/or membrane redistribution and has also been identified as the foetal stem cell marker AA4 (Nepomuceno *et al.*, 1997; Dean *et al.*, 2000; Kim *et al.*, 2000; Lovik *et al.*, 2000). Another study demonstrated that C1q did not show enhanced binding to C1qRp-transfected cells and a soluble recombinant C1qRp-FC chimera failed to interact with immobilised C1q (McGreal *et al.*, 2002). It has also been suggested that complement receptor 1 (CR1) or CD35 in addition to acting as a receptor for C3b also acts as an MBL-receptor (Ghiran *et al.*, 2000). However, CR1 has been described on all peripheral blood cells (except platelets), follicular dendritic cells, peripheral nerve fibers, and glomerular podocytes but has yet to be described on human endothelial cells (Ross, 1992). We also failed to show its presence on HUVEC (unpublished data). Collard *et al.* demonstrated by confocal microscopy that LPS increased the expression of CD35 by HUVEC but this was predominantly intracellular (Collard *et al.*, 1999). The quest for MBL receptors continues and the mechanism by which MBL inhibits CD62E expression upon stimulation with meningococci remains to be identified.

The severity of meningococcal disease appears to be correlated with the degree of inflammatory cell activation with the highest mortality seen in patients with excessive levels of proinflammatory cytokines at presentation (Waage *et al.*, 1987). There have been a number of reports that have described modifications of the

inflammatory response by MBL. The first, by Soell *et al.* (1995), showed inhibition of tumour necrosis factor (TNF) output by monocytes in response to streptococcal rhamnose polymers, a molecule related to the lipopolysaccharide of Gram-negative bacteria. Another report showed that MBL decreased TNF release in response to a cryptococcal membrane glycoprotein (Chaka *et al.*, 1997), and yet another showed that MBL enhanced TNF release after monocytes were exposed to the intact organism, *Candida albicans* (Ghezzi *et al.*, 1998). Interestingly, MBL has been shown to reduce phagocytosis of *Candida* (Kitz *et al.*, 1992). Similarly, increasing concentrations of MBL caused increasing release of TNF and interleukin 6 (IL6) from monocytes responding to promastigotes of *Leishmania chagasi* (Santos *et al.*, 2001). The addition of high concentration of MBL (>6 μg/ml) profoundly decreased the production of interleukin IL-6, IL-1β, and tumour necrosis factor – α by monocytes in response to meningococci, whereas lower concentrations (<5 μg/ml) enhanced the production of IL-6 and IL-1β (Jack *et al.*, 2001). More recently Sprong *et al.* (2004) demonstrated that MBL at a concentration of 2.5 μg/ml augmented IL-1β and IL-10 production by human PBMCs induced by meningococci.

Changes in adhesion molecule expression lead to changes in neutrophil adhesion to vascular endothelial cells, and therefore the potential role for MBL to delay this process or to regulate the underlying mechanisms could have profound implications for the severity of disease processes.

Chapter 7 The role of MBL in opsonophagocytosis

7.1. Introduction

In 1976 an opsonic defect was described in about 25% of children suffering from frequent and often serious infections (Soothill *et al.*, 1976). The defect was manifested by a failure of the serum to promote complement deposition and phagocytosis of the yeast *Saccharomyces cerevisiae* (Levinsky *et al.*, 1978). There was no deficiency in any of the known complement components and no other immunological abnormalities could be demonstrated. Subsequent investigations have shown that this opsonising defect occurs in 5-7% of the population (Kerr *et al.*, 1983) and in 1989 the defect was found to be caused by deficiency of mannan-binding lectin (Super *et al.*, 1989).

Phagocytosis can be mediated by MBL in two ways: via complement activation, or through an intrinsic effect of MBL itself. The original report by Kuhlman *et al.* (1989) appeared to show that MBL alone attached to *Salmonella enterica* serovar Montevideo was sufficient to increase uptake of the organism by neutrophils and monocytes and enhance the function of bacterial killing mechanisms. More recently, it has been shown that the same organism was not to be taken up by neutrophils unless they were first stimulated by fibronectin (Ghiran *et al.*, 2000). MBL was only able to increase phagocytosis of *Salmonella* by unstimulated neutrophils when bacteria were coated with sub-optimal amounts of IgG. MBL was found to increase the uptake by macrophages of *Cryptococcus neoformans* opsonised with serum or immunoglobulin, using a slide assay of phagocytes placed on the top of immobilised MBL and then offered to a phagocytic target (Levitz *et al.*, 1993). A similar slide assay was used to show the increase of phagocytosis of C4b- or Ig-coated erythrocytes by MBL (Tenner *et al.*, 1995).

Additional opsonic factors do not always seem to be necessary for MBL to increase phagocytosis. MBL alone enhances influenza A uptake by neutrophils (Hartshorn *et al.*, 1993) and mycobacterial uptake by macrophages (Polotsky *et al.*, 1997), although the latter could be part of the infection strategy of this group of organisms. Jack *et al.* (2001) have shown a direct effect of MBL on the uptake of *N.meningitidis* by neutrophils, monocytes and macrophages, although such uptake by neutrophils is unlikely to be significant in terms of organism killing (Drogari-Apiranthitou *et al.*, 1997). In a further investigation of neisserial phagocytosis Jack

et al. (2005) found that MBL improves the uptake of these bacteria by enhancing the ability of macrophages to internalise bacteria bound to the macrophage membrane. MBL does not appear to be involved in the initial binding of organisms to the phagocytic membrane (Jack *et al.*, 2005).

This study was designed in order to evaluate the effect of exogenous MBL on the opsonophagocytosis of *Staphylococcus aureus* by neutrophils.

Acknowledgment

An experimental work in this chapter which investigated the deposition of C4, factor B, C3b and iC3b on *S. aureus* was done by Dr. O.Neth.

7.2 Materials and methods

7.2.1 Growth and preparation of organisms

S. aureus strain 6571 was obtained form the National Collection of Type Cultures and was stored on beads at -70°C. Organisms from beads were cultured overnight at 37°C in 6% CO_2 on blood agar and subcultured once before use. Immediately before each experiment organisms were suspended in HBSS at 1-3x10^8 organisms/ml (measured as an absorbance of 0.5 at 540 nm). We have previously shown that this organism binds a high level of MBL (Neth *et al.*, 2000).

7.2.2 Human sera

Serum was available for study from three MBL deficient donors (RC, NS and DM). The MBL haplotypes and serum MBL levels of these individuals were as follows: donor RC, HYPD/LYPA (150 ng/ml); donor NS, LYPB/LXPA (150 ng/ml) and donor DM, LYPB/LYPB (<150 ng/ml). Levels of MBL were determined using a commercially available AntibodyShop ELISA kit (Denmark) as described previously in Chapter 2.5.

7.2.3 FITC labelling of organisms

S. aureus was labelled with FITC as previously described for anti-MBL antibodies in Chapter 2.6.3. FITC-labelled *S. aureus* was stored in 10% glycerol broth in 50 µl aliquots at –70°C and before use the concentration was evaluated using the Miles and Misra technique (Miles *et al.*, 1938).

7.2.4 Conjugation of biotin to antibodies

Biotin conjugation of anti-C4d was carried out using biotinamidocaproate N-hydroxysuccinimide ester (Sigma, Poole, UK) at a ratio of ester to antibody of 7.5:1 as described in Chapter 2.6.2.

7.2.5 Purification of neutrophils

Neutrophils were purified on a Ficoll/Histopaque gradient as described previously (Jansen *et al.*, 1998). 10 ml of heparinised whole blood were diluted 1/2 with HBSS. The diluted blood had been carefully layed over 7 ml of Ficoll-Paque

gradient and centrifuged for 20 min at room temperature at 400xg without a brake. The mononuclear cells were carefully removed from the plasma-Ficoll-Paque interface and washed in 10 ml of HBSS twice. Erythrocytes were lysed by the addition of 10 ml of sodium chloride for 10 minutes. Neutrophils were washed in HBSS and the viability count was determined by staining the cell suspension with trypan blue. Phagocytosis was evaluated using a multiplicity of infection (MOI) of 5 organisms to 1 neutrophil.

7.2.6 C4 deposition on *S. aureus* NCTC6571

50 μl aliquots of organisms at a concentration of 1-3x10^8 organisms/ml were spun in Eppendorf tubes at 9470x g for 2 min. Supernatants were removed and the pellets resuspended in 25μl of 10% MBL-deficient serum to which were added different amounts of purified human MBL (to give 0.3, 0.6, 1.2, 2.5, 5 and 10 μg/ml final concentration). The mixtures were then incubated for 5 min at 37°C. The reaction in each tube was stopped by adding 1 ml of ice cold HBSS containing 1% para-formaldehyde and pelleted organisms were washed twice in 200 μl HBSS. A mixture of FITC-conjugated anti-MBL and biotinylated anti-C4d (Quidel, Betchworth, UK) was added to give a final concentration of 4 μg/ml for each and the mixture incubated for 45 min on ice. Streptavidin phycoerythrinPE-Cy 5 (Pharmingen, Cowley, UK) at a concentration of 0.66 μg/ml was added and the organisms incubated as before. Suspensions were spun at 9470 x g for 2 min. The supernatants were removed and the pellets washed with 200 μl of HBSS. The samples were then fixed using equal volumes of PBS/FACSfix. Flow cytometry was performed on a FACS-Calibur at low flow rates using CellQuest software (Becton Dickinson, Cowley, UK). Organisms were selected on the basis of size and granularity and analysed by two colour flow cytometry.
Binding of C4 was evaluated on at least three occasions. A negative control comprising organisms processed in the same way but in the absence of serum and MBL was included in every assay.

In subsequent experiments, following the initial centrifugation of organisms, the supernatants were removed and the pellets resuspended in 25μl of either 10%

MBL-deficient serum or 10% MBL-deficient serum supplemented with 5 μg/ml purified MBL. The mixtures were then incubated for 30 sec, 1, 2, 3 or 5 min at 37°C. Thereafter the reaction was stopped in each tube and the assay completed as above.

7.2.7 C4/Bb deposition on *S. aureus* NCTC6571

C4b and Bb fragments were bound to organisms when sample mixtures were incubated as described above for 0.5, 1 and 3 min at 37°C in the presence of 5 μg/ml of purified MBL. The reaction was stopped as before by ice cold HBSS/1 % para-formaldehyde and the preparations washed twice in 200 μl HBSS. A mixture of FITC-conjugated anti-Bb and biotinylated anti-C4d (Quidel) was added at a final concentration of 4 μg/ml for each and incubation was allowed to proceed for 45 min on ice. Streptavidin phycoerythrinPE-Cy 5 (Pharmingen) at a concentration of 0.66 μg/ml was added and the organisms incubated as before. Suspensions were spun at 9470 x g for 2 min and the pellets washed as described above. Samples were processed, fixed as described above and analysed by flow cytometry as described above.

7.2.8 iC3b/C3b deposition on *S. aureus* NCTC6571

iC3b and C3b fragments were bound to organisms when sample mixtures were incubated as described above for 1, 3 and 5 min at 37°C. The reaction was stopped as before using ice cold HBSS/1 % para-formaldehyde and the organisms washed twice in 200 μl HBSS. Aliquots of 25 μl of mouse anti-human iC3b (tissue culture supernatant used at 1/20 dilution) or 25 μl of mouse anti-human C3b (tissue culture supernatant used at 1/50 dilution) were added into separate tubes corresponding to each time point. Both mouse antibodies were kindly supplied by Dr. Sunita Gulati (The Maxwell Finland laboratory for Infectious Diseases, Boston, USA). The mixtures were then incubated for 45 min on ice. A preparation of FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulins (Dako, High Wycombe, UK) was added at a final concentration of 25 μg/ml to each sample and the mixture incubated for 40 min at 4°C. Samples were then processed, fixed and analysed by flow cytometry as described above.

7.2.9 Opsonophagocytosis of *S. aureus*

50 µl aliquots of FITC labelled organisms at a concentration of $1x10^7$ organisms/ml were transferred to sterile 96 well plates (Immulon 2, Dynex Technologies, Chantilly, VA). 25µl volumes of HBSS, 10% MBL-deficient serum or 10% MBL-deficient serum supplemented with 5 µg/ml MBL were added to separate wells and the mixture incubated for 1, 3 or 5 min at 37°C on a shaking table. The reaction was stopped at each time point by adding 200 µl of ice cold HBSS (calcium and magnesium free (cmf) and the mixtures spun for 10 min at 1500 x g. The supernatants were discarded and the pellets resuspended in 50 µl ice cold HBSS (cmf). Neutrophils ($2x10^6$/ml) in 50 µl aliquots were added and the mixtures incubated for 10 min at 37°C on a shaking table. After the addition of 100 µl ice cold HBSS (cmf) the samples were spun for 10 min (1500 x g). The pellets were resuspended in 100 µl PBS and fixed using 100 µl Cell-fix (Becton Dickinson) diluted 1/10 in ddH_2O before analysis by flow cytometry. Uptake of the FITC-labelled *S. aureus* was evaluated by determining tbe median fluorescence intensity (MFI) of the neutrophil population. To estimate the proportion of internalised as opposed to adherent bacteria, extracellular fluorescence was quenched by adding trypan blue (2 mg/ml) for 10 min. The samples were then re-analysed by flow cytometry and MFI values determined. In one series of experiments heat inactivated serum (56°C for 30 min) was used in addition to unheated serum and in another series of experiments neutrophils were purified from four different healthy adult donors in order to minimise any donor specific effect.

7.2.10 Statistical analysis

To compare the amount of C4 deposited over the course of the experiment, the area under the curve for each experiment with or without MBL was calculated and compared by paired t-tests.

7.3 Results

7.3.1 C4 deposition on *S.aureus*

In order to investigate the activation of complement by the MBL-MASP complex bound to *S. aureus* NCTC 6571, the organisms were incubated with 10% serum from three different MBL deficient donors, to which a preparation of 5 μg/ml of MBL was added. C4 deposition was evaluated by flow cytometry and, as shown in Figure 7-1, was detected by 0.5 minutes incubation in serum. The amount of C4 appeared to be enhanced by MBL at this time point and subsequent time points. C4 deposition was maximal at 5 minutes in the presence and absence of MBL, after an initial peak at 1 min, but after 5 minutes, the amount of C4 deposited on *S.aureus* declined more markedly in the presence of MBL. MBL significantly enhanced the total amount of C4 deposited over the 10 minute incubation period in serum (p=0.002).

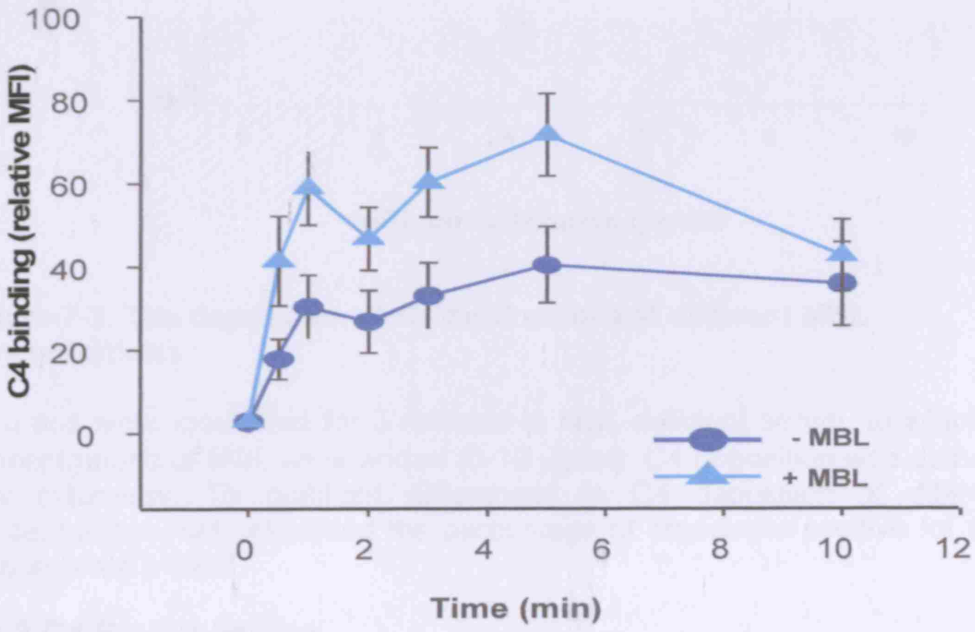


Figure 7-1. Time course of C4 deposition on *S.aureus*.
5 μg/ml of MBL were added to MBL-deficient serum from three different donors and the C4 deposition was determined by flow cytometry at different time points. Binding of C4 is expressed as relative MFI based on the maximum MFI obtained in each experiment. Error bars indicate ± SEM.

To determine the dosage effect of MBL on C4 activation, organisms were incubated for 5 minutes in MBL-deficient serum, to which different concentrations of MBL were added (0-10 μg/ml) (Figure 7-2). MBL at concentrations of 0.6 μg/ml or above enhanced C4 deposition on *S. aureus* ($p<0.05$, unpaired t-test) and it appeared to reach a maximum at 5 μg/ml.

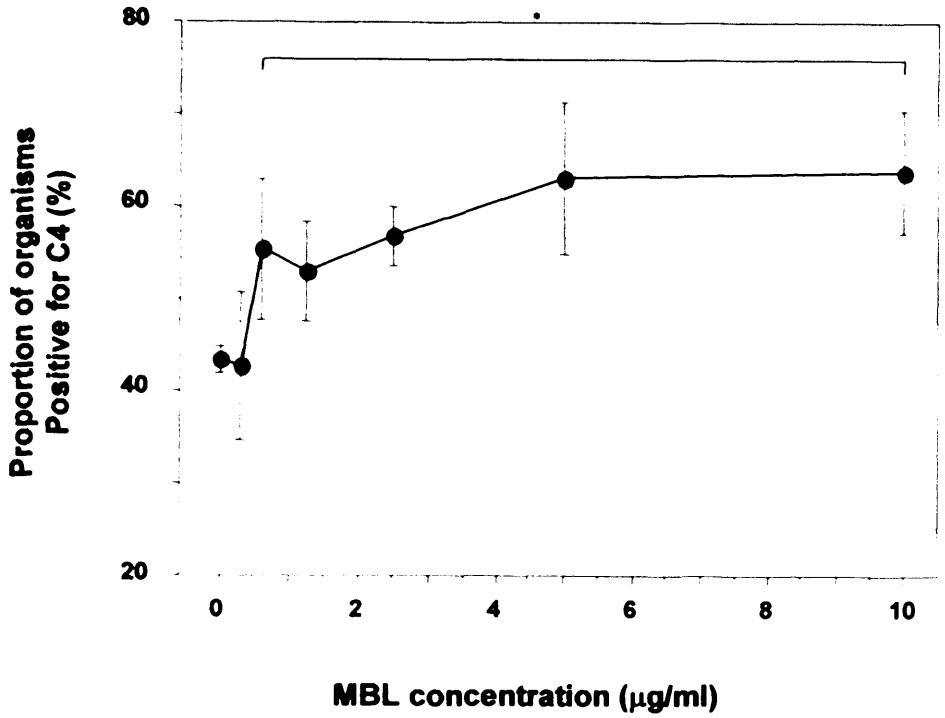


Figure 7-2. The deposition of C4 on *S.aureus* at different MBL concentrations.

S.aureus were incubated for 5 minutes in MBL-deficient serum, to which different concentrations of MBL were added (0-10 μg/ml). C4 deposition was determined by flow cytometry. To highlight differences in C4 deposition at different MBL concentrations, we calculated the percentage of organisms positive for C4. Error bars indicate ± SEM.

7.3.2 C4/Bb deposition

The relationship between C4 and Bb deposition was analysed at three time points by flow cytometry using 10% serum from an MBL deficient donor (RC) or the same serum to which was added exogenous MBL (final concentration 5 μg/ml).

In the absence of MBL approximately 35 % of the organisms showed evidence of C4 binding after 30 secs and the addition of exogenous MBL increased this to more than 60 % (Figure 7-3). At this time there was negligible Bb deposition on the organisms. When samples were analysed after 1 minute the data were similar.

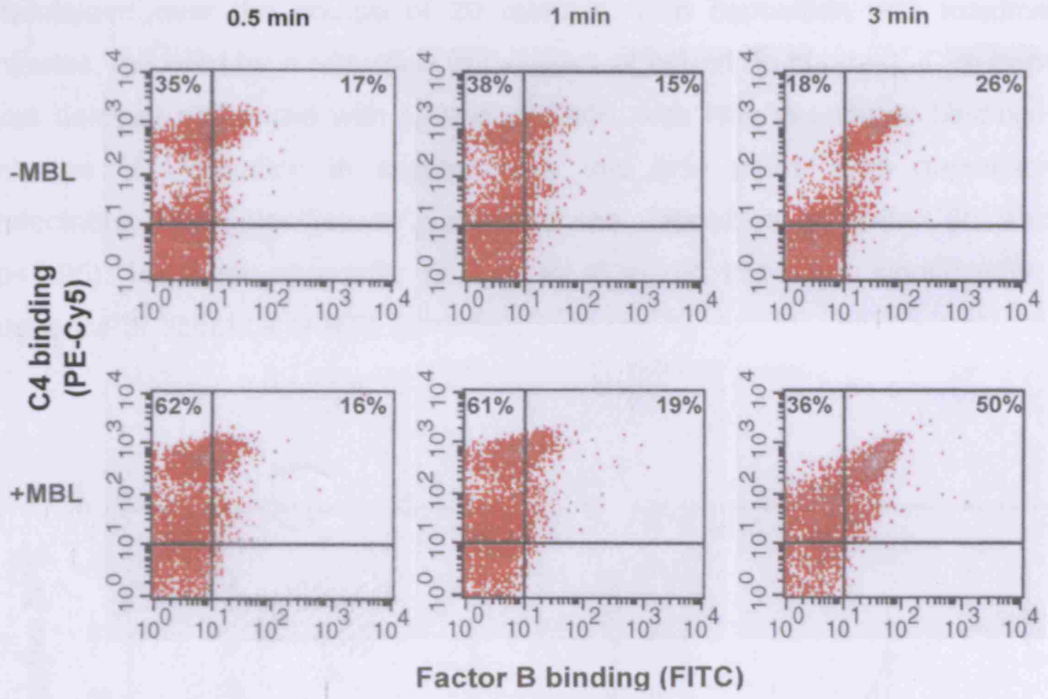


Figure 7-3.Deposition of C4 and factor B on *S. aureus*.

Organisms were incubated with 10% MBL-deficient serum (donor RC) or MBL-deficient serum supplemented with 5 μg/ml MBL. The plots shown are representative density plots of C4 against factor B at the different time points from 10 different experiments.

When samples were removed and analysed after incubation for 3 minutes, approximately 25% of the organisms showed evidence of both C4 and Bb binding in the absence of MBL. However, when the MBL deficient serum was supplemented with MBL more than 50% of organisms stained positively for both ligands.

7.3.3 C3b/iC3b deposition

S. aureus NCTC 6571 was incubated with 10% serum from the MBL deficient donor, RC, or the same serum supplemented with 5 μg/ml MBL. C3b deposition was detected after incubation with serum for 1 minute (Figure 7-4), and as for C4, there was an enhancement of deposition in the presence of MBL that was maintained over the course of 20 minutes. C3b deposition was maximal at 3 minutes, followed by a reduction in the amount bound. In contrast, iC3b deposition was delayed compared with C3b deposition, with little detectable binding until 3 minutes of incubation in serum. After this time point, iC3b deposition was detectable. MBL significantly enhanced the deposition of both C3b and iC3b ($p<0.05$). However, the ratio of C3b to iC3b did not differ significantly in the presence or absence of MBL ($p>0.05$).

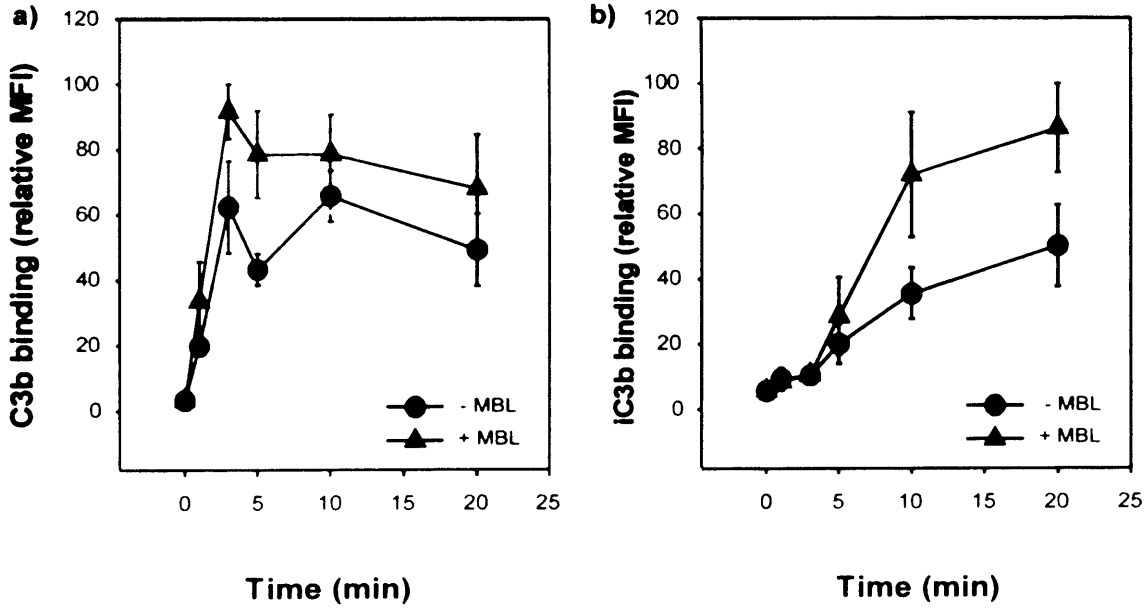


Figure 7-4. The deposition of C3b and iC3b on *S. aureus* NCTC657 using 10% serum from an MBL-deficient donor (RC).

Exogenous (5 μg/ml) MBL was added to the serum and the reactions were stopped at 0, 1, 3, 5, 10, and 20 minutes with ice cold buffer. The binding of C3b (Figure a) and iC3b (Figure b) was determined separately. Binding of each moiety is expressed as relative MFI based on the maximum MFI obtained in each experiment. Error bars indicate ± SEM.

7.3.4 Uptake of organisms by phagocytes

In the present study, flow cytometry was used to investigate the effect of MBL on the opsonophagocytosis of *Staphylococcus aureus* by polymorph nuclear cells. Using flow cytometry it was possible to detect a distinct population of neutrophils based on forward and side scatter. Events were collected on the basis of size and granularity, as shown in Figure 7-5.

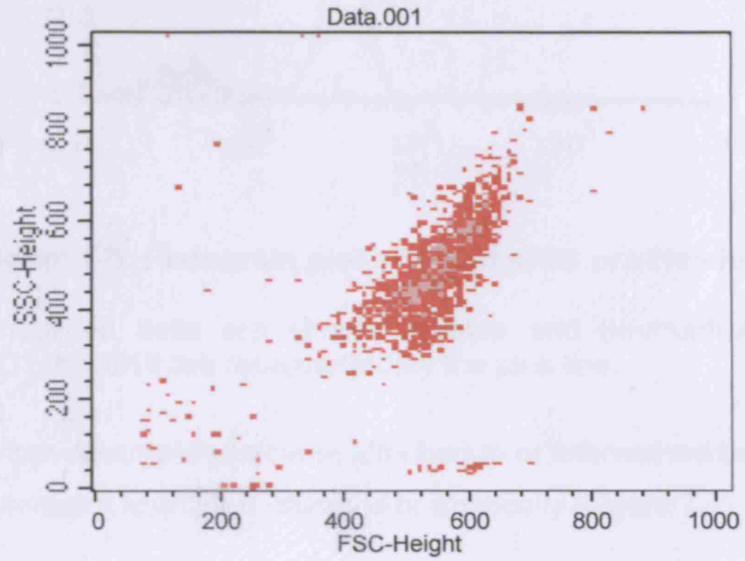


Figure 7-5. Neutrophil density plot.

Cells were identified as neutrophils by positive CD11b/CD18 staining as shown in Figure 7-6.

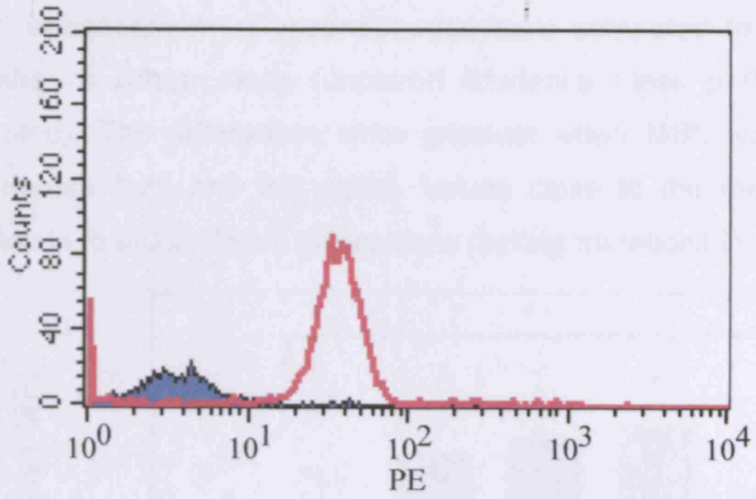


Figure 7-6. Histogram plot of neutrophils positive for CD11b/CD18 staining.

Unstained cells are shown in blue and neutrophils which were positive for CD11b/CD18 are represented by the pink line.

When neutrophils became attached to or internalised bacteria the profile of the flow cytometry histogram changed dramatically (Figure 7-7).

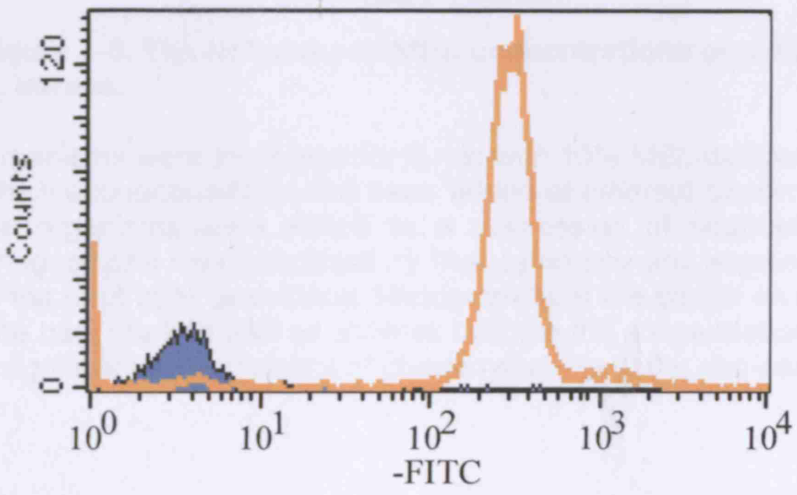


Figure 7-7. Histogram plot of opsonophagocytosis of *S.aureus* by neutrophils.

The blue peak represents neutrophils with no associated *S.aureus* organisms whereas the orange line represents neutrophils which internalised FITC-labelled bacteria.

As shown in Figure 7-8 the addition of increasing MBL concentrations resulted in an enhancement of opsonophagocytosis compared to that observed using MBL deficient serum alone (unpaired Student's t-test, $p<0.05$ for all concentrations tested). The differences were greatest when MBL was used at concentrations between 1.25 and 2.5 μg/ml, values close to the median concentration of the protein found in British Caucasians lacking mutations in exon 1 of the MBL2 gene.

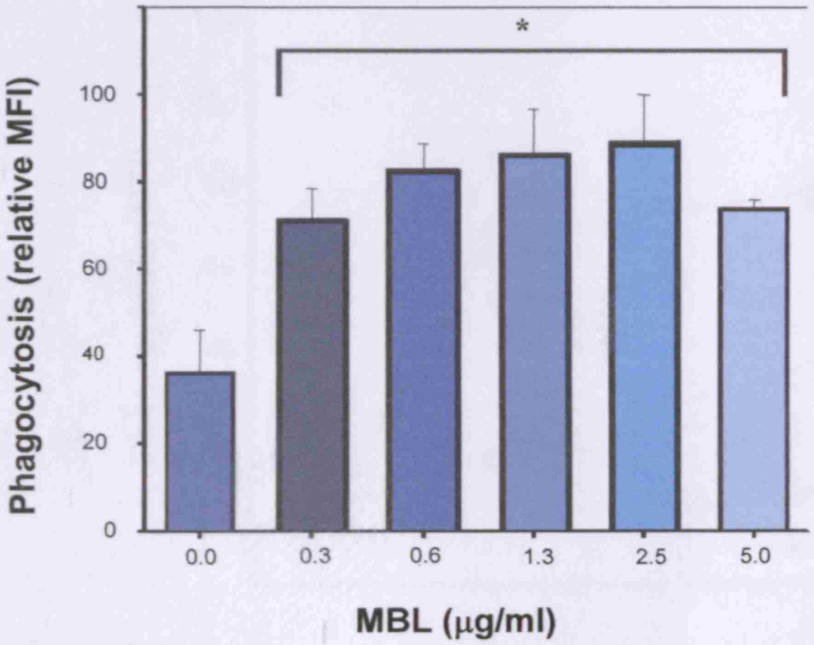


Figure 7-8. The influence of MBL concentrations on opsonophagocytosis of *S. aureus*.

Organisms were incubated for 5 min with 10% MBL-deficient serum (donor RC), to which exogenous MBL had been added at different concentrations. After washing, the organisms were added to a suspension of neutrophils at an MOI of 5:1. Phagocytosis was quantified by flow cytometry and expressed as the relative MFI of the neutrophil population. Histogram bars are shown as mean MFI ±SEM (n=3). The bars marked with an asterisk indicate the concentrations of MBL-MASP giving a significant enhancement of phagocytosis ($p<0.05$, non-paired *t* test).

When FITC labelled bacteria were incubated with 10% MBL deficient serum (RC) and 10% heat inactivated MBL deficient serum for different periods of time (0-5 min) it was found that the addition of MBL (5 μg/ml) enhanced opsonisation in both normal and heat inactivated serum with major differences apparent within 1 min (Figure 7-9).

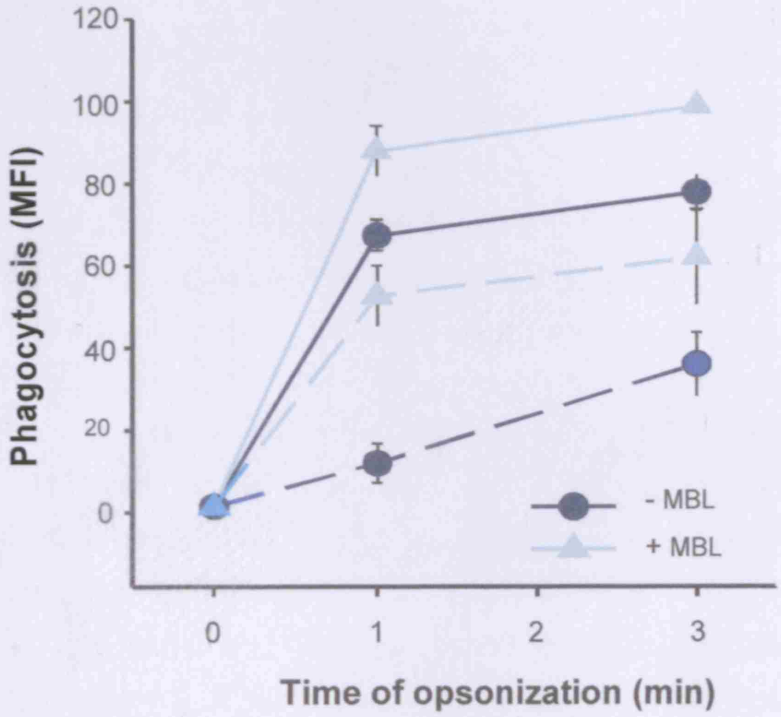


Figure 7-9. Opsonophagocytosis of *S.aureus*.

FITC-labelled *S.aureus* was incubated with MBL-deficient serum with or without MBL (5 μg/ml) for different time periods. After washing, organisms were added to a suspension of neutrophils at an MOI of 5:1 and phagocytosis was stopped after 10 min. The uptake of organisms by neutrophils was analyzed by flow cytometry and expressed as relative MFI. Continuous lines show experiments using MBL-deficient serum, with the dashed lines showing experiments using the same serum heat inactivated at 56°C. The dark blue line represents experiments with 10% serum from an MBL-deficient donor; the light blue line represents experiments in which bacteria had been incubated with 10% serum from an MBL-deficient donor supplemented with MBL-MASP (5 μg/ml). Data plotted as mean MFI±SEM (n=3).

The results illustrated in Figure 7-9 were subsequently confirmed in five separate experiments using neutrophils from four different donors. The addition of exogenous MBL to the MBL deficient serum significantly increased

opsonophagocytosis as evaluated at the 5 minutes time point (p=0.001, two way Friedman analysis of variance).

The patterns of opsonophagocytosis observed following the addition of trypan blue, though variable between different donors, suggested that MBL both enhanced the attachment of organisms to neutrophils and also promoted internalisation (data not shown).

7.4 Discussion

In the present study, MBL significantly enhanced the binding of C4 (the first cleavage product of MBL-MASP activation) to *S.aureus* over the course of 10 minutes in sera from MBL-deficient donors. In every case there was evidence of enhanced C4b deposition over and above that generated by the classical and alternative pathways of the different donors. This phenomenon is, therefore, apparently independent of the individual antibody titres against *S. aureus* antigens. The increase in C4 deposition was detectable when the lectin was present at concentrations above 0.6 μg/ml.

Activation of the lectin pathway will generate a C3 convertase that will produce C3b fragments which can combine with factor B to generate the C3 convertase of the alternative pathway. Dual-colour flow cytometry was used in the present study to investigate the relationship between classical or lectin pathway activation (C4 binding) and the binding of factor B. MBL generated C3 convertase is able to recruit the alternative pathway amplification loop, thereby enhancing C3 cleaving activity and the generation of C3b opsonin. The addition of MBL markedly increased the proportion of organisms positive for C4 after only one minute (79% compared to 53% in the absence of MBL). At this time point there was a similar proportion of organisms staining positive for factor B in the presence or absence of MBL. At 3 min, the total proportion of organisms positive for C4 in the absence of MBL had declined, but there was an increased association with factor B. In contrast, in the presence of MBL, C4 deposition had not decreased at 3 min and factor B association was approximately doubled (more than 50% of the organisms were $C4^+/Bb^+$ in the presence of MBL compared to only 26% in the absence of the collectin). It should be noted that the percentage of organisms positive for C4 at 5 min was somewhat lower than observed at earlier time points. C4 deposition occurred at a higher level with a less rapid decline in the presence of MBL. This effect of MBL on the kinetics of complement activation and the apparent loss of C4 are similar to the previous results with *Neisseria meningitidis* where a more rapid deposition of C4 and C5b-9 was associated with increased killing of these Gram negative organisms in serum (Jack *et al.*, 2001). These new observations suggest that MBL-initiated complement activation directly recruits the alternative pathway

amplification loop. Indeed the lectin pathway may contribute substantially to the formation of the C3bBb alternative pathway C3 convertase. In this respect it was notable that there was no appreciable association of factor B with organisms that had not also bound C4, which may suggest that direct initiation of the alternative pathway was not occurring at this concentration of serum.

C3b covalently bound to microbial surfaces is converted to iC3b as a result of enzymic cleavage by Factor I at two sites in the α chain. Although there are distinct receptors for C3b and iC3b (CR1 (CD35) binds C3b whereas CR3 (CD18/CD11b) and CR4 (CD18/CD11c) bind iC3b) the wide distribution of these receptors ensures effective opsonophagocytosis through both the C3b and iC3b routes.

In this study MBL clearly enhanced the generation of C3b detectable on the surface of staphylococci and may have a similar effect on iC3b deposition which occurs later. The appearance of C3b fragments was detectable after only a 1 min incubation in serum, as has been described elsewhere (Tofte *et al.*, 1980; Gordon *et al.*, 1988), with detectable iC3b appearing at 3-5 min. It has been shown that there are differences between bacteria in the rate of conversion of C3b to iC3b. Gordon and co-workers (Gordon *et al.*, 1988) found that when bacteria were opsonised in 50% pooled human serum C3b deposition and cleavage to iC3b occurred rapidly and that the proportions of C3b, iC3b and C3d on *S. aureus* were 17%, 64% and 19%, respectively, whereas in the case *E. coli* the proportions were 53%, 44% and 2%. However, in 10% serum, a smaller fraction of the C3b bound was converted to iC3b and our findings would be consistent with this observation. MBL apparently did not alter the proportion of C3b converted to iC3b overall; it simply increased the amount of C3b deposited, which led to an increased amount of iC3b. MBL may influence the subsequent fate of generated C3b in an organism-specific fashion but the consequences of altering the balance between C3b/iC3b are difficult to predict. However, one possibility is that such changes may alter the pathways through which bacteria can interact with phagocytes. This may influence subsequent inflammatory pathway activation and thereby influence the onset and progression of bacterial infections (Jack *et al.*, 2001; Santos *et al.*, 2001; Soell *et al.*, 1995; Jack *et al.*, 2001).

The studies described in this Chapter were aimed at determining the effect of increased complement activation on phagocytosis of *S. aureus* by neutrophils. It had been observed that the binding and internalisation of organisms incubated with MBL-deficient serum for short time periods could be enhanced by the addition of purified MBL in a dose dependent manner. This correlated with the increased deposition of C3 that had been observed in the presence of MBL. Interestingly, our study demonstrated an increase in phagocytosis with MBL even when the serum was heat inactivated. This suggests that MBL may have an intrinsic effect on phagocytosis that is not linked to its ability to enhance complement activation. The mechanisms by which MBL may influence phagocytosis are controversial. MBL has been reported to act as a direct opsonin (Christensson *et al.*, 1985; Colque-Navarro *et al.*, 2000) and as an agent which modifies the efficiency of other pathways such as Ig-mediated or CR1-mediated phagocytosis (Ryding *et al.*, 2002; Peterslund *et al.*, 2001). Since our experiments were performed in the presence of serum it is possible that other factors such as immunoglobulins, may have been involved in enhancing the uptake of bacteria by the neutrophils (Tofte *et al.*, 1980; Peterslund *et al.*, 2001; Ryding *et al.*, 2002).

One caveat applicable to all of the findings reported here is that MBL is not the sole activator of the MASP family. Matsushita *et al.* (2002) have shown that serum ficolins, lectins with collagen-like and EGF-like domains, are able to activate complement through an association with MASP-1, MASP-2 and MAp19 which functionally parallels the MBL-MASP system (Matsushita *et al.*, 2000; Matsushita *et al.*, 2002). It seems likely that there is a competition between MBL and ficolins for the various serum proteases of the MASP family. It cannot be excluded that the preparation of MBL used in this study was completely free of ficolin. However, dot blot analysis failed to generate evidence in support of this notion. Furthermore, the purification procedure used to isolate MBL involved the use of EDTA and mannose to elute the protein from affinity columns, and these characteristics also argue against ficolin contamination. However, the serum of MBL-deficient donors would be expected to contain ficolins and therefore the background activation observed in all MBL-deficient donors studied would represent a summation of Ab-mediated classical pathway activation and ficolin-mediated activation. At the same time the

conclusion that the various enhanced activities observed were ascribable to the MASP system of complement activation still stands and the data available suggests that MBL-MASP complexes are probably much more efficient than Ficolin-MASP complexes in the activation of C4 (Suankratay *et al.*, 1998). Further clarification of the stoichiometry of the interactions between the two lectins and the various MASPs is urgently required if we are to fully understand the complexities of the human complement system.

Chapter 8 Conclusions

Mannose-binding lectin is a vital and versatile component of the innate immune system. Deficiency of the protein due to genetic polymorphisms has been associated with several infectious diseases. However, recent studies propose that the functions of MBL may extend beyond its classic role as a first-line host-defence molecule. The work presented in this thesis provides some further insights into the possible contribution of MBL in human disease.

8.1 The binding of MBL to micro-organisms

MBL binds to a range of carbohydrates, activates the classical complement pathway in an Ab-independent manner, and is believed to act directly as an opsonin (Kuhlmann *et al.,* 1989). Although the binding to monosaccharides has been well defined (Holmskov *et al.*, 1994), binding to complex saccharides, in particular to microbial structures, is poorly understood. MBL preferentially recognises glucans, lipophosphoglycans and glycoinositol phospholipids that contain mannose, glucose, fucose, or N-acetylglucosamine as their terminal hexose (Weis *et al.*, 1992). The first study to demonstrate that the variation in the surface structures of pathogens may be important for MBL binding was done by Kawakami *et al.* (1982). These investigators identified a serum factor, Ra reactive factor (RaRF), which bound to and killed particular forms (Ra-chemotypes) of *Salmonella* (Kawakami *et al.*,1982; Kawakami *et al.*,1984). RaRF has since been shown to be identical to MBL (Matsushita *et al.*, 1992). These reports provide the earliest evidence of the effect of LPS structure and composition on MBL binding and function. More recent studies have confirmed the effect of LPS structure on MBL attachment to both *Salmonella* enterica serovar *Typhimurium* (Devyatyarova-Johnson *et al.,* 2000) and the human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis* (serogroups B and C) (Jack *et al.*, 1998; Jack *et al.*, 2000). MBL binding patterns to *Salmonella* were similar to those with *Neisseria*, in that organisms with no O-antigen (Ra or rough chemotype) showed MBL binding but organisms with O-antigen (smooth chemotype) exhibited little or no binding (Devyatyarova-Johnson *et al.*, 2000). The data in this thesis supports previous work that has shown the importance of LPS in determining MBL binding.

The organisms 44/76 and *siaD Neisseria meningitidis* and *Helicobacter pylori* (smooth chemotype) did not show MBL binding at any concentration studied. Also variations in lipid A structure in all organisms studied did not influence the MBL binding pattern. However, variations in LPS structure did dramatically change the level of MBL binding. For example the *ltgB* mutant of *Neisseria meningitidis*, which fails to add galactose to GlcNAc in the outer core shows a significant level of MBL binding, whereas others such as mutant *rfaK*, which failed to add heptose to KDO1-lipid A, showed no MBL binding. These results are consistent with previous findings (Devyatyarova-Johnson *et al.*, 2000) and highlight the critical role of LPS structure as a determinant of MBL binding. All our finding have been recently confirmed (Dumestre-Perard *et al.*, 2006).

The other conclusion that can be drawn from these experiments is that the presence of a particular terminal oligosaccharide (even if it has affinity for MBL) in the LPS structure may not be sufficient to support MBL binding to the bacterium. A very recent study (Krarup *et al.*, 2005) has demonstrated that environment- and growth-dependent variations of bacterial surface structures may influence the binding potency of the lectin to bacteria. It is clear that the exposure of potential binding sites to MBL is not always predictable and binding will be determined by the presence of a range of different components within the bacterial cell wall. For example, a recent study has shown that the presence of excess galactose on the bacterial surface can support MBL binding, in spite of its low affinity for MBL (Konishi *et al.*, 2006). Other components that can influence MBL binding include bacterial capsule, peptidoglycan, which consists mainly of N-acetylglucosamine and is a major cell wall component of gram-positive bacteria (Nadesalingam *et al.*, 2005), phospholipids, nucleic acids (Palaniyar *et al.*, 2004) and non-glycosylated Opa proteins on the surface of *Neisseria meningitidis* (Estabrook *et al.*, 2003). This array of potential binding sites is perhaps the reason why MBL is effective as a first line of defence against bacterial infection.

8.2 What defines MBL deficiency?

Early studies of the relationship between MBL levels and disease susceptibility relied on quantification of MBL in serum or plasma. Many of these studies utilised MBL assays that were developed within the laboratory of the investigators. Now, there are several commercial suppliers of MBL kits which include Sanquin reagents, Amsterdam, the Netherlands; Hycult Biotechnology, Leiden, the Netherlands; AntibodyShop, Copenhagen, Denmark; Dobeel Corp in South Korea and Dade Behring (although the latter has now been removed from the market). A formal comparison of these methods has not been performed. This is potentially problematic as it is now recognised that some assays are more sensitive than others when measuring lower oligomeric forms (Lipscombe *et al.,* 1995; Garred *et al.*, 2003). A recent study demonstrated that four commercially available kits are selectively sensitive for the higher oligomeric forms of MBL, while special combinations of antibodies can also detect mutant forms of MBL (Frederiksen *et al.,* 2006). In Chapter 4 of this thesis, a comparison was made between three assays: an in house ELISA assay, the MBL Oligomer ELISA kit by AntibodyShop and the Dade Behring immunonephelometric research procedure assay. It was shown that these assays do indeed give very different results. To assess what they are actually measuring matched sera from genetically typed individuals were utilised. All assays gave similar results for individuals with a WT genotype. However for individuals heterozygous and homozygous for MBL2 exon 1 polymorphisms, the Dade Behring assay in particular, but also to a lesser extent the in house ELISA procedure, gave higher readings than the Antibody shop assay. Therefore it can be concluded that the latter assay provides the best serum indication of MBL2 haplotypes.

In addition to MBL2 genotype and phenotype, MBL function may also be clinically relevant. A number of assays, most commonly using a mannan coating and anti-MBL antibodies, have been used to estimate the capacity of MBL to activate the MBL-lectin pathway. This may be important for identifying individuals with MASP-deficiencies (MASP-2 deficiency had been recently described by Stengaard-Pedersen *et al.*, 2003). Recently, a kit for estimating the functional activity of the

entire MBL pathway has been manufactured commercially (Wieslab, Lund, Sweden). Functional assessment of the lectin pathway may be more clinically relevant than measuring MBL level and/or genotype alone (Dommett *et al.*, 2006). Future studies are needed to clarify which of various assays should be used in clinical practice.

Numerous studies have been performed to assess the clinical impact of MBL deficiency and disease. Even after 15 years of work in this area, there is as yet no consensus as to what MBL level signifies deficiency. Individuals with identical haplotypes may differ by 10-fold in their MBL levels (Steffensen *et al.*, 2000). As explained above this is in part due to the variety of assays used in these studies and the presence of low molecular weight MBL in individuals with structural variants (Lipscombe *et al.*, 1995; Garred *et al.*, 2003). However this is not the whole story. It has become apparent that few studies exist in which levels in healthy individuals have been related to genotype. The previous studies have used blood donors and as such it is impossible to know how representative that population really is. The ALSPAC cohort is one of the first unselected general cohorts studied and could give us a better guide as to the genotype-phenotype relationship in an unselected population. From the results of this study, it would appear that there is a very tight genotype-phenotype correlation. Only 3 patients with a wild type genotype had MBL levels below 1300ng/ml. All heterozygous individuals apart from A/B and A/C with an X promoter had levels between 400 and 1300 ng/ml. Nearly all homozygotes had levels of less than 75ng/ml with the XA/B or C having levels between 75 and 400 ng/ml. At least in the resting state in healthy individuals, phenotype can provide a very accurate assessment of genotype. This may not always be the case in children who are unwell. Although the levels of MBL in children with cystic fibrosis who carried wild type or one structural variant allele are comparable to our control population, the levels of MBL in homozygous children with CF were 10 times higher.

8.3 MBL has a complex role in human disease

Cystic fibrosis provides an example of a clinical condition where MBL appears to play a role but not simply as a result of enhanced bacterial killing. In this study, MBL haplotypes and phenotypes did not appear to be related to reduced lung function in children with cystic fibrosis. This contrasts with the effect of MBL in adults (Davies *et al.*, 2004). It had been suggested that an association between MBL status and severity of lung function impairment in cystic fibrosis may be age dependent (Muhlebach *et al.*, 2006) because adults with cystic fibrosis living today have been treated differently during childhood compared to today's pediatric population (Olesen *et al.*, 2006). How MBL is operating in cystic fibrosis is still unclear. In contrast with Gabolde *et al.*, other studies (Garred *et al.*, 2003; Davies *et al.*, 2004) demonstrated that MBL does not influence susceptibility to *P. aeruginosa* or *B. cepacia*. This is despite results from an *in vitro* study demonstrating high levels of MBL binding to clinical isolates of *B. cepacia* and subsequent activation of complement (Davies *et al.*, 2000). There is no consensus as to whether MBL binds to *Pseudomonas aeruginosa*. Neth et al. (2000) failed to detect MBL binding to *Pseudomonas aeruginosa* by FACS analysis. Kuipers *et al.* (2003) demonstrated MBL-dependent complement activation by *P. aeruginosa* in their assay using erythrocyte lysis. The most recent study has also demonstrated that MBL binds to *P. aeruginosa* using a solid phase ELISA type procedure and a suspension phase binding assay. They confirmed that MBL binding to this organism was not detected by FACS analysis (Moller-Kristensen *et al.*, 2006). Another study demonstrated that patients not colonised by *P.aeruginosa* were mainly colonised by *S. aureus* and furthermore, in these patients, MBL-deficient genotypes were associated with impaired lung function (Carlsson *et al.*, 2004).

Taken together, these findings might suggest that complement-mediated clearance of bacteria may not be the major mechanism through which MBL is operating in the CF lung. Nevertheless, levels of binding that are insufficient to activate complement-mediated killing may be sufficient to modulate the host inflammatory response to a microbial insult. MBL deficient mice were more susceptible to *P. aeruginosa* infection due to higher bacterial load and decreased

opsonophagocytosis of bacteria by effector cells (Moller-Kristensen *et al.*, 2006). In addition to the much lower levels of circulating protein available to reach the airways in O/O individuals, mutant forms of the protein may be more susceptible to degradation by the matrix metalloproteases (Butler *et al.*, 2002) that are present in abundance in the CF ASF, and, thus, MBL-deficient individuals may be doubly compromised. This hypothesis can be examined further by measuring levels of MBL and inflammatory cytokines directly in CF bronchoalveolar lavage samples.

Previous studies have indicated that MBL deficiency is associated with increased susceptibility to meningococcal disease. In our study we did not confirm these previous findings. This was not due to the frequency of variants in the meningococcal group, which was similar to that described previously. It was due to the higher rate of variants in uninfected children in our study compared to the Hibberd cohort. This suggests that at most, MBL deficiency plays only a minor role in enhancing susceptibility to disease caused by this organism. There are however, good reasons to think that MBL could play a role in determining the severity or outcome of the disease.

Previous studies have shown that MBL activates complement on the meningococcus, and can enhance bacterial killing (Jack *et al.*, 2001). As such MBL could be expected to contribute to the control of bacterial numbers in the bloodstream. Recent studies have shown that bacterial numbers are a good guide to assessing outcome in meningococcal disease (Øvstebø *et al.*, 2004). This may be in part because of the inflammatory consequences of bacterial killing. In a study by Lehner *et al.* (1992), of a C6 deficient patient, it was the addition of FFP that induced cytokine release and clinical manifestations of sepsis indicating that complement is involved in the inflammatory response to bacterial infections. MBL mediated complement activation is no exception as the recent studies looking at ischaemia reperfusion injury demonstrate (Jordan *et al.*, 2001; de Vries *et al.*, 2004).

MBL also appears to influence the production of cytokines in *vitro* in a complement independent fashion which could be important in meningococcal disease. In a

whole blood model, MBL modulated the production of IL1β and IL6 while in peripheral blood mononuclear cells MBL enhanced IL1β and IL10 production (Sprong *et al.*, 2004). This effect appears to be dependent on bacterial numbers and so might be expected to be relatively more important in patients with higher bacterial loads.

On their own, MBL genotypes were not associated with altered risk of death, although there were trends towards a reduced risk of death in YAYO or YOYO individuals. High bacterial load was associated with an increased risk of mortality which presumably relates to the higher levels of inflammatory mediators being released in these individuals. However, this relationship between bacterial load and death only existed in patients with normal levels of MBL. In individuals with reduced levels of MBL, bacterial load did not predict the likelihood of death. Only 10% of YAYO patients with high bacterial loads died compared to 35% of patients with normal levels of MBL. MBL could be involved in modifying the cytokine response to infection. In particular, in this study there was some evidence that MBL had an effect on patients with IL1 polymorphisms previously shown to be associated with increased mortality. Further work is required to understand how MBL is operating in bacterial sepsis

Further evidence that MBL is involved in modulating inflammation comes from the work presented in Chapter 5 which has shown that genetic polymorphisms resulting in MBL deficiency are associated with increased susceptibility to severe sepsis and septic shock in adults. The mechanisms involved are unclear but again may be related to inflammatory modulation. MBL also binds to apoptotic cells and facilitates their phagocytosis by macrophages (Ogden *et al.*, 2001). Lower MBL levels may prevent effective removal of dead cells and bacteria which again will have inflammatory consequences. Further work is required in this area.

8.4 MBL is more than a complement activating protein

Our study demonstrated that MBL enhanced the deposition of different complement components on the surface of *S.aureus* and opsonophagocytosis of the same microorganism by neutrophils. Our observations received further support when MBL-null mice were later shown to be highly susceptible to *S. aureus* infection compared to wild type mice (Shi *et al.*, 2004). The same study demonstrated that MBL not only acts as an opsonin, but also stimulated a proinflammatory response. MBL-null mice had decreased levels of IL-6 and TNF-α at 2 hours after bacterial inoculation compared to wild type mice. This resulted in sepsis and death of all MBL-null mice by 48 hours with high levels of IL-6 and TNF-α. This correlated with studies showing that *S. aureus* preincubated with serum from MBL-null mice was ineffective in cytokine release by macrophages (Shi *et al.*, 2004). Another recent study has demonstrated that MBLxC3 null mice were more susceptible to *S. aureus* infection than mice deficient in C3 only (Takahashi *et al.*, 2005). This finding suggested a new distinctive host defence mechanism of MBL that is independent of complement, at least against *S. aureus*. MBL was initially recognised as an opsonin (Super *et al.*, 1989) and later identified as the initiator of the lectin complement pathway with MASPs (Matsushita *et al.*, 1992; Thiel *et al.*, 1997). However, it has not been clear whether MBL simply amplifies the complement cascade or has its own role that is independent of the complement pathway. The mechanism whereby MBL act as a direct opsonin has yet to be investigated in detail. There is a possibility that MBL acts in parallel to complement. This effect could be either synergistic or additive, since the effect of MBL deficiency seemed to increase that of C3 deficiency. This independent activity of MBL most likely operates via collectin receptors but the identity of the latter remains unclear. Candidate molecules have included CD91/calreticulin (Ogden *et al.*, 2001; C1q receptor (Nepomuceno *et al.*, 1997), and/or CD21/CD35 receptor (Ghiran *et al.*, 2000). Another study suggested that MBL enhanced phagocytosis of A. *otitidis* via scavenger receptor A (Konishi *et al.*, 2006). MBL also may regulate other receptors, either directly by facilitating accessibility of ligands or indirectly by binding to the collectin receptors that may further modulate other receptors (Jack and Turner, 2003).

It is difficult to explain how MBL has such a dramatic effect in such a wide variety of conditions. As mentioned in the previous section there is increasing evidence that MBL may do more than activate complement. It has been shown that MASP-free MBL and C1q recognise a shared receptor on the endothelial cell surface (Oroszlán *et al.*, 2006). This study demonstrated the presence of only one receptor on the cell surface of HUVECs that had been previously implicated in interactions with C1q and MBL, namely calreticulin. However, preliminary attempts to inhibit the binding of MBL to HUVECs failed (Oroszlán *et al.*, 2006). The work presented in Chapter 6 demonstrated that MBL reduced *N.meningitidis* induced CD62E expression by endothelial cells. The mechanisms were unclear but this was not uniform for all adhesion molecules as ICAM-1 levels were not affected. Further studies are ongoing to try and understand how MBL is operating in this situation. It is interesting that MBL also reduces the expression of the adhesion molecules, CD11b and CD62L by neutrophils (Jack *et al.*, 2001). Again the reasons for this are unclear but may relate to the receptors engaged on the surface and within the phagolysosomes within the neutrophils as has been shown for macrophages (Jack *et al.*, 2005).

8.5 MBL Replacement Therapy

MBL replacement therapy was first attempted when fresh frozen plasma was given to patients to correct the opsonic defect (Miller *et al.*, 1968; Soothill and Harvey. 1976). Since then plasma derived purified MBL has been safely given to many patients (Valdimarsson *et al.*, 1998). The development of recombinant MBL is also at the phase 1/2 trial stage. Infusions of MBL have good safety profile and have a reasonably long half-life (Petersen *et al.*, 2006). It is still unclear exactly which patients will benefit from MBL replacement therapy but possible uses include elective MBL replacement therapy in bone marrow transplantation or acute treatment of septic patients with MBL functional deficiency.

Appendix I

Flow cytometry settings

HUVEC		
Detector	Voltage	Amp gain
Forward scatter	E-1	1.00
Side scatter	347	1.00
Fluorescence-1 (FITC)	396	1.00
Fluorescence-2 (PE)	417	1.00
Threshold	200	
Bacteria		
Forward scatter	EO1	1.00
Side scatter	430	1.00
Fluorescence-1 (FITC)	630	1.00
Fluorescence-2 (PE)	570	1.00
Threshold	200	

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